Free radicals and reperfusion-induced arrhythmias in the isolated rat heart

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FREE RADICALS AND REPERFUSION-INDUCED ARRHYTHMIAS IN THE ISOLATED RAT HEART

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Research is a cost or an investment;
far from delivering guaranteed results,
it is a highly speculative, highly uncertain
effort that requires the greatest managerial
competence to produce results.

Peter F. Drucker.

For all sad words of tongue or pen,
The saddest are these:
"It might have been!".

John G. Whittier.

To Mum and Dad.

With your love, support
and encouragement you
made this possible.

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ABSTRACT

This study investigated the involvement of oxygen free radicals in reperfusion arrhythmogenesis using the isolated rat heart. By studying the movement of potassium using the radioactive tracer ion $^{86}Rb^{+}$, it was shown that both reperfusion and free radical generation brought about a substantial efflux of $^{86}Rb^{+}$. This response was potentially arrhythmogenic and occurred at a time when reperfusion arrhythmias were observed.

Whilst the free radical scavenger glutathione was shown to be anti-arrhythmic and to attenuate the reperfusion-induced efflux of $^{86}Rb^{+}$, several other anti-free radical interventions proved ineffective. The importance of oxygen free radicals to reperfusion arrhythmogenesis is therefore questionable. Furthermore, glutathione exhibited a pronounced coronary dilator action, although by comparing the effects of other sulphydryl agents and vasodilator drugs with those of glutathione, it was shown that coronary dilation did not significantly contribute to the drug's antiarrhythmic action. A reduction of $^{86}Rb^{+}$ efflux following reperfusion appeared to be more closely associated with drug-induced coronary dilation than with a reduction of arrhythmias.

A possible source of oxygen free radicals in the heart is the enzyme xanthine oxidase. The present study demonstrated that the xanthine oxidase inhibitor allopurinol is antiarrhythmic but not through inhibition of xanthine oxidase, since its active metabolite oxypurinol was without effect on the incidence of reperfusion arrhythmias. The location and activity of xanthine oxidase were investigated in cell cultures using luminol-enhanced

chemiluminescence. In rat cells, xanthine oxidase exists predominantly in the vasculature, thus precluding a role for the enzyme in reperfusion-induced damage since the half-lives of radicals are probably too short to allow diffusion from the vasculature to the myocyte.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 REPERFUSION-INDUCED ARRHYTHMIAS:DEFINITION AND CLINICAL RELEVANCE

Approximately 50% of deaths that occur in the western world in people over the age of 20 years are the direct result of cardiovascular disease (Pantridge *et al*,1975). In the majority of cases, death is sudden and unexpected, and whilst ventricular arrhythmias resulting from myocardial ischaemia are thought by some to be the underlying cause (Corr and Witkowski, 1984; Luchessi, 1984), post-mortem examinations often fail to show evidence of coronary occlusion (Sheridan, 1987). Observations that link transient changes in coronary artery tone to the development of arrhythmias have directed a considerable amount of research into the phenomenon of reperfusion-induced arrhythmogenesis (Manning and Hearse, 1984). Reperfusion-induced arrhythmias, as defined by Manning and Hearse (1984), are disturbances of heart rhythm that arise as a consequence of the total or partial restoration of coronary flow to a previously ischaemic region of heart tissue. The clinical relevance of reperfusion arrhythmias is immediately apparent given the increasing use of thrombolytic therapy and mechanical coronary angioplasty. The success of such techniques is often assessed by the development of arrhythmias (Goldberg *et al*, 1983). Whilst the involvement of reperfusion arrhythmias to sudden cardiac death is difficult to substantiate, the possibility remains that it is an important contributory factor.

1.2 FACTORS THAT INFLUENCE THE DEVELOPMENT OF REPERFUSION ARRHYTHMIAS

1.2.1 Ischaemic Duration

Several studies have demonstrated an inverse relationship between the incidence of reperfusion-induced arrhythmias and the duration of the preceeding period of ischaemia (Balke *et al*,1981; Penny and Sheridan, 1983; Crome *et al*, 1983). The vulnerability of isolated rat hearts to ventricular fibrillation (VF) exhibits a bell-shaped relationship with ischaemic duration (Manning and Hearse, 1984). The highest incidence of VF occurs during reperfusion of hearts rendered ischaemic for 15 min. A similar relationship was demonstrated in the anaesthetised rat, with the exception that the bell-shaped curve was displaced to the left, showing a maximum vulnerability to reperfusion arrhythmias after only 5 min of ischaemia. Evidence exists for such a relationship in humans and has been reviewed by Corr and Witkowski (1983).

Findings such as these suggest that the development of arrhythmogenic conditions during myocardial ischaemia is time-dependent. Ischaemia-induced changes that may contribute to the development of arrhythmias are considered later in more detail.

1.2.2 The Rate of Reperfusion

Investigations of the relationship between reperfusion rate and the development of reperfusion arrhythmias have yielded equivocal

results. Petropoulos and Meijne (1964) were able to reduce the incidence of VF by reperfusing globally ischaemic isolated guinea pig hearts with low flow rates. In the same heart model, Sheridan (1987) was unable to reproduce these results, but demonstrated a delayed onset of VF with gradual reperfusion. In an isolated rat heart model of regional ischaemia, Zakaria (1985) reduced the incidence of reperfusion arrhythmias by lowering the rate of reperfusion. Speculative explanations for these effects (Corr and Witkowski, 1983; Manning and Hearse, 1984) include the facilitation of a slower, more uniform washout of toxic metabolites formed during ischaemia, and a less abrupt reoxygenation process resulting in a more gradual restoration of ionic homeostasis.

The general concensus of opinion is that rapid changes of the substrate and ionic environment across the reperfused region may contribute to the development of reperfusion arrhythmias (Corr and Witkowski, 1983).

1.2.3 Collateral Flow

Variations between species in the degree of existing collateral circulation, and the ability to develop a collateral supply under ischaemic conditions, may affect the vulnerability of hearts to reperfusion arrhythmias. The degree of recovery afforded by reperfusion, in terms of cardiac performance and electrophysiology, is improved when myocardial necrosis is limited, which is itself achieved when collateral flow is high (Przyklenk *et al*, 1986). However, reperfusion-induced recovery may also be arrhythmogenic,

as will be considered later (section 1.3.2).

For experimental models of myocardial ischaemia/reperfusion, the choice of species is critical. The hearts of some animals possess a high degree of collateral flow and in the guinea pig, for example, it is impossible to induce regional ischaemia because of this (Schaper, 1984).

1.3 PUTATIVE MECHANISMS OF REPERFUSION ARRHYTHMOGENESIS

1.3.1 Electrophysiological Changes Associated with Ischaemia and Reperfusion

During myocardial ischaemia, the electrophysiological changes that occur in ischaemic tissue are non-uniform. Progressive reductions of action potential duration, resting membrane potential and the maximum rate of depolarisation (V_{max}) occur throughout the ischaemic region but at different times (Downar *et al*, 1977). The heterogeneity of depolarisation is further demonstrated by a fractionation of electrical activity into multiple, widening spikes. Endocardial to epicardial electrical conduction is delayed (Murdock *et al*, 1980) and the effective refractory period within the ischaemic zone has been shown to both shorten and lengthen (Russell and Oliver, 1978), again indicating inhomogeneity of electrical activity. Some studies (Downar *et al*, 1977; Russell *et al*, 1977) have demonstrated the development of post-repolarisation refractoriness.

Some ischaemia-induced changes in action potential characteristics are normalised upon reperfusion. Rapid recovery of the amplitude and upstroke velocity is achieved within seconds of reperfusion, particularly when the ischaemic period is short (Penny and Sheridan, 1983). Other indices of electrical activity take longer to recover. Re-synchronisation of depolarisation is only achieved after several minutes of reperfusion (Kaplinsky *et al*, 1981), and the effective refractory period is reduced below ischaemic values (Naimi *et al*, 1977) and so increases the heterogeneity of refractoriness between normal and previously ischaemic tissue.

The heterogeneity of electrical activity during ischaemia therefore appears to be exacerbated by reperfusion. The restoration of coronary flow to an ischaemic region paradoxically results in the genesis of ventricular arrhythmias through changes in action potential characteristics and electrical conduction. The contribution of these changes to possible arrhythmogenic mechanisms is discussed below.

1.3.2 Arrhythmias Resulting from Re-entry Mechanisms

Circus movement re-entry results from the unidirectional block of impulse conduction, which allows retrograde re-excitation of tissue proximal to the area of block (Rosen *et al*, 1987). To allow the proximal tissue to recover excitability following the initial impulse, there must also be a slower tissue conduction velocity and/or shortened refractoriness before re-entry can occur, both of

which are observed during early reperfusion, when VF is generated (Corr and Witkowski, 1983).

It is worthy of note that re-entry does not necessarily lead to VF (Sheridan, 1987). Dysrhythmias resulting from this mechanism may be organised to activate the whole ventricular mass, appearing as VT. Only when the electrophysiology of cardiac tissue is sufficiently disturbed (for example, when an activation wavefront interrupts an incompletely depolarised area of tissue) is synchronous activity lost, leading to fibrillation. This situation is more likely to occur with an increasing heterogeneity of electrophysiological conditions, such as those brought about by ischaemia, upon reperfusion and during reperfusion-induced recovery of the action potential. According to Manning and Hearse (1984), the heterogeneity of *recovery* may be particularly relevant to re-entry mechanisms following reperfusion. The authors suggest that the heterogeneity of the developing *injury* is crucial to the development of *ischaemic* arrhythmias, whereas reperfusion arrhythmias may also be a consequence of the heterogeneity of cellular recovery processes. Evidence in support of this hypothesis is mainly circumstantial. As mentioned earlier, the highest incidence of reperfusion-induced VF was shown to occur in the isolated rat heart after 15 min of ischaemia (Crome *et al*, 1983). With longer ischaemic durations, the incidence of VF declines. It has been suggested (Hearse, 1983) that reversible cellular injury occurs with early reperfusion of ischaemic tissue and that reversibility of damage lends itself to re-entry mechanisms. Early reperfusion (i.e. reperfusion following short (10-15 min) periods

of ischaemia) in isolated rat hearts predisposes to the development of VF (Lubbe *et al*, 1978; Woodward and Zakaria, 1983; Manning and Hearse, 1984).

It therefore appears likely that life-threatening reperfusion arrhythmias (VF) are brought about predominantly by re-entry processes. This argument is supported by several authors (Corr and Witkowski, 1983; Manning and Hearse, 1984; Sheridan, 1987), although the *initiation* of reperfusion arrhythmias by re-entry mechanisms remains a matter of controversy (Sheridan, 1987).

1.3.3 Enhanced Automaticity

Cardiac automaticity is the ability of hearts to initiate action potentials as a result of spontaneous phase 4 depolarisation. This automatic activity predominates in the sinoatrial node and results from the diminution of an outward repolarisation current (carried mainly by potassium ions) simultaneous with the initiation of an inward current (carried by sodium and calcium ions). The intracellular accumulation of a positive charge depolarises the cell to a threshold potential and an electrical impulse ensues.

Epicardial mapping experiments in dogs (Ideker *et al*, 1981) have shown that reperfusion-induced VF is initiated shortly after synchronous electrical activation of tissue within the border of the ischaemic/non-ischaemic zone. It is possible that VF in these experiments is caused by rapid automaticity, although the precise activation sequence was not assessed owing to the small number of

recording sites confined to the epicardium only. An attempt to overcome this problem was recently described by Pogwizd and Corr (1987). Using a three-dimensional computerised mapping system to simultaneously record from 232 intramural sites in the cat heart *in vivo*, the authors observed VT arising from the subendocardial border zone. In 75% of cases, tachycardia was accompanied by non-continuous activation of impulses that were uninterrupted, suggesting the participation of nonre-entrant mechanisms. Tachycardia in the remaining 25% was thought to involve intramural re-entry in the subendocardium. Transition of VT to VF, and the maintenance of arrhythmias during reperfusion, were associated with both nonre-entry and intramural re-entry processes. The transition period was associated with rapid acceleration of tachycardia through nonre-entrant mechanisms, causing conduction delay and so precipitating re-entrant cicuits. No definitive conlusions were drawn as to the nature of the nonre-entrant processes, although Pogwizd and Corr suggested the involvement of abnormal automaticity or triggered activity (see below).

Further evidence for the role of enhanced automaticity in reperfusion arrhythmias comes from the work of Sheridan and colleagues (1980). Increases in idioventricular rate during reperfusion were observed in cats at a time when ventricular tachyarrhythmias were most intense. Accelerated idioventricular rates have also been observed in humans following intracoronary thrombolysis (Goldberg *et al*, 1983). Sheridan's group attributed the rise in rate to stimulation of alpha-adrenoceptors by catecholamines released during reperfusion, since depletion of

myocardial catecholamines countered the rate increase, whereas administration of methoxamine (an alpha-receptor agonist) reversed the electrophysiological effects of catecholamine depletion (Sheridan *et al*, 1980). An increase in alpha-receptor number during ischaemia, proposed by Corr *et al* (1981), may also contribute to the electrophysiological changes observed on reperfusion.

Alternative mechanisms for the generation of tachyarrhythmias are early and late afterdepolarisations. Every action potential is proceeded by oscillations of membrane potential (Rosen *et al*, 1987). These afterdepolarisations may occur before completion of phase 3 repolarisation (resulting from either or both a decrease in the outward repolarising potassium current and an increase in the inward current carried by sodium or calcium), when they are termed "early" afterdepolarisations. Alternatively, "delayed" afterdepolarisations may be observed as oscillations of membrane potential occurring on completion of repolarisation following an action potential. Delayed afterdepolarisations are seen following digitalis toxicity (Rosen *et al*, 1973). Inhibition of sodium-potassium ATPase by the cardiac glycoside leads to an increase in the inward sodium current and subsequent calcium overload through sodium-calcium exchange (Kass *et al*, 1978). The release of calcium from sarcoplasmic reticulum and mitochondria was also demonstrated. Enhanced calcium influx triggers an inward current, carried mainly by sodium ions, which results in depolarisation (Rosen *et al*, 1987).

Both early and delayed after-depolarisations may reach threshold

potential and initiate tachyarrhythmias. Both rely on the intracellular accumulation of calcium ions which may lead to a rise in the extracellular potassium concentration. Calcium overload has been observed following reperfusion (Hearse, 1977; Ferrari *et al*, 1986) and is a possible trigger for the generation of afterdepolarisations, although little evidence is available which correlates the time of calcium overload with the time at which reperfusion arrhythmias occur (Manning and Hearse, 1984). Extracellular potassium accumulation does occur during ischaemia (Hirche *et al*, 1980), but no conclusive relationship has been demonstrated between extracellular potassium concentration and reperfusion arrhythmogenesis.

Although definitive data still need to be produced which link reperfusion arrhythmias with enhanced automaticity, the possibility remains that the latter initiates arrhythmias. The literature reviewed above tends to support the concept that VF following reperfusion is *initiated* by automatic mechanisms and *sustained* by re-entry processes or by a combination of re-entry and nonre-entry mechanisms.

1.4 REPERFUSION-INDUCED PERTURBATIONS OF IONIC HOMEOSTASIS

The generation and propagation of ventricular arrhythmias is ultimately the result of electrical abnormalities caused by disturbances of normal ionic movements. Normal cardiac function, and in particular the cardiac action potential, is regulated by several ionic species. The following paragraphs briefly consider

the ischaemia and reperfusion-induced changes in the homeostasis of two such ions; calcium and potassium.

1.4.1 Calcium

The depolarisation phase of the ventricular action potential is mediated, in part, by a slow inward current carried by calcium and (to a lesser extent) sodium ions (Morad and Tung, 1982). This current, together with an efflux of potassium ions (see below), is also responsible for the maintenance of the plateau phase, and in this way determines the duration of the action potential. The critical involvement of calcium in the determination of action potential characteristics, coupled with observations of substantial reperfusion-induced calcium overload (Hearse, 1977; Ferrari *et al*, 1986), favour the proposal that reperfusion arrhythmias are inextricably linked with altered calcium ion fluxes. Delayed afterdepolarisations may be precipitated by a high intracellular calcium concentration, and this mechanism has been suggested as a possible cause of reperfusion arrhythmias (Corr and Witkowski, 1983). In support of this, an increase in extracellular calcium concentration (which elevates the intracellular calcium concentration) causes a shortening of the action potential plateau, an increased rate of phase 0 depolarisation and a prolongation of atrioventricular conduction (Gettes, 1981). The slow conduction of action potentials arising from these changes would favour circus movements of excitation and may lead to re-entry arrhythmias (Parratt, 1982).

Despite these convincing hypotheses, there is little evidence of a role for calcium overload in reperfusion arrhythmogenesis. Many of the proposed mechanisms fall short by virtue of the timing of calcium overload in relation to the timing of VF. The use of calcium entry blocking drugs, such as verapamil, has been shown to both reduce reperfusion arrhythmias (Bergey *et al*, 1984) and have little effect on arrhythmia incidence (Kane *et al*, 1984). As yet, there is no definitive proof that calcium overload is a trigger for VF resulting from the early stages of reperfusion (for reviews, see Manning and Hearse, 1984 and Opie and Coetzee, 1987).

1.4.2 Potassium

Potassium, the most abundant intracellular cation, is important for the maintenance of the cardiac cell resting membrane potential. At rest, the negative potential of the intracellular compartment is achieved mainly by virtue of the movement of potassium out of the cell along its concentration gradient. This is facilitated by a high membrane permeability to potassium ions. The potassium gradient is maintained by an inwardly rectifying, voltage-dependent potassium channel and the electrogenic sodium-potassium pump (Morad and Tung, 1982). During the plateau (phase 2) of non-nodal tissue action potentials, there is a large efflux of potassium, the magnitude of which depends upon the intracellular calcium concentration. There is however, no net change in membrane potential because of inward rectification (which effectively reduces membrane potassium conductance) and a positive inward current carried by calcium and sodium ions. Finally, repolarisation

of the membrane is achieved when potassium conductance rises simultaneous with a reduction of the inward movements of calcium and sodium.

Many studies have been concerned with the changes in extracellular potassium ion concentration during ischaemia, with very little emphasis on changes associated with reperfusion. During ischaemia, potassium accumulates in the extracellular space of the ischaemic region and exhibits a triphasic time course (Hill and Gettes, 1980; Hirche *et al*, 1980; Weiss and Shine, 1982). Within 5 to 10 min of coronary occlusion, concentrations rapidly rise to between 10mM and 12mM. Levels plateau for approximately 15 min and then begin to rise slowly until the potassium concentration may exceed 30mM. Hill and Gettes (1980) have shown that the first two phases are rapidly reversed by reperfusion, but the changes in the final, slowly rising phase are not reversible and may correspond to the time at which irreversible cell damage occurs.

Ischaemia-induced reductions of the resting potential and rate of rise of phase 0 and the increase in post-repolarisation refractoriness have been attributed to the above changes in potassium concentration (Gettes, 1987). Similarly, the initial enhancement of excitability and conduction during ischaemia are thought to be a consequence of extracellular potassium accumulation. Each of these responses can be reproduced by raising the potassium concentration of bathing or perfusing solutions (Gettes, 1987) and are associated with the complications resulting from hyperkalaemia, which may lead to cardiac dysrhythmias

(Schwartz, 1978).

Other ischaemia-induced changes could exacerbate the electrophysiological changes associated with potassium accumulation. Gaspardone and colleagues (1986) postulated an ionic-dependent loss of potassium from the myocardium during ischaemia, since hypoxic perfusion of rabbit septa induces an efflux of (undefined) anions resulting in an electrochemical gradient along which potassium ions are extruded. The intracellular acidosis associated with ischaemia (Hirche *et al*, 1980), when induced in isolated rabbit hearts perfused with hyperkalaemic solutions (Weiss and Shine, 1981), quantitatively reproduced ischaemia-induced alterations to action potential duration and conduction time (Gaspardone *et al*, 1986).

As previously mentioned, changes in the extracellular potassium concentration during reperfusion have received little attention. The above review illustrates the importance of potassium in maintaining the normal action potential in cardiac non-nodal cells and the possible role of abnormal potassium fluxes in precipitating the aberrant electrophysiological characteristics of ischaemia. It is conceivable that alterations of extracellular potassium concentrations during reperfusion, and the likely ionic heterogeneity caused by such changes, predisposes hearts to reperfusion-induced arrhythmogenesis.

1.5 BIOCHEMICAL AND METABOLIC FACTORS IMPLICATED IN ARRHYTHMOGENESIS

The following is a brief account of biochemical factors which may participate in the genesis of ischaemic and reperfusion arrhythmias. It is not the purpose of this thesis to provide an extensive review of each research area. The information given below is therefore intended to be an illustration of the wide-ranging complexity of putative endogenous arrhythmogenic agents and hopefully provides a source of reference to more detailed appraisals.

1.5.1 Prostanoids

The release of products of the oxidation of arachidonic acid has been demonstrated during myocardial ischaemia (Coker *et al*, 1981). The balance between the local generation of thromboxne A_2 and prostacyclin from platelets, leucocytes and the blood vessel wall has been implicated as a major contributory factor in ischaemia and reperfusion arrhythmogenesis (Parratt, 1987). In patients with acute myocardial infarction, plasma levels of thromboxane A_2 and 6-keto PGF_{1alpha} (the major metabolite of prostacyclin) were elevated and reduced respectively (Friedrich *et al*, 1985). Inhibition of thromboxane synthesis with steroids and non-steroidal anti-inflammatory drugs by Karmazyn (1985), reduced the release of creatine phosphokinase and LDH associated with reperfusion damage. Similar studies by Parratt and Coker (1985) demonstrated a protective effect against arrhythmias by inhibition of thromboxane

release or by thromboxane receptor blockade, and by an enhancement of prostacyclin generation.

The mechanism by which certain prostanoids upset normal cardiac electrophysiology is not known. Direct actions on the vasculature and platelets are likely to be involved. Prostaglandins of the E series, and prostacyclin itself, have been shown to inhibit the release of noradrenaline from adrenergic nerve terminals by restricting the neuronal uptake of calcium (Hedqvist,1976; Lanier and Malik, 1985), and so may protect against ischaemia-induced noradrenaline overflow (see section 1.5.3). The possible role of prostanoids in free radical-mediated cardiac damage will be discussed later (section 1.6).

1.5.2 Free Fatty Acids and Lysophospholipids

Ischaemia associated with reduced coronary flow and reduced oxygen supply inhibits the beta-oxidation of free fatty acids (FFA) in the mitochondria of ischaemic cells (Neely and Feuvray, 1981). As a result, beta-oxidation substrates (long chain acyl coenzyme A and long chain acyl carnitine) accumulate, particularly if levels of circulating FFA are high. Acyl coenzyme A and acyl carnitine exert a detergent action on membranes which may account for their inhibitory effects on membrane-bound enzymes such as calcium-ATPase (Pitts *et al*, 1978; Corr *et al*, 1984) and sodium-potassium ATPase

(Owens *et al*, 1982).

FFA are stored in the myocyte by incorporation into phospholipids (Corr *et al*, 1984). Enhanced activity of membrane-bound or lysosomal phospholipases, calcium-dependent enzymes responsible for the catabolism of phospholipids, has been proposed to account for elevated levels of phospholipids during ischaemia (van der Vusse *et al*, 1982). More experimental evidence favours the reduced catabolism of lysophospholipids by membrane-bound lysophospholipases, the activities of which are reduced under ischaemic conditions (Gross and Sobel, 1982) and inhibited by long chain acyl coenzyme A, long chain acyl carnitine and FFA (Gross and Sobel, 1983). Lysophospholipids are amphiphilic molecules capable of insertion into cell membranes, wherein normal membrane function may be compromised (Corr and Sobel, 1983). Electrophysiological derangements, similar to those seen during ischaemia, have been produced in isolated canine Purkinje fibres by the application of lysophospholipids (Corr *et al*, 1979). VF resulting from intracoronary infusion of lysophosphatidylcholine has been demonstrated in the anaesthetised cat (Bentham, 1986).

The role of the above changes during coronary reperfusion is less apparent. Reperfusion-induced calcium overload could enhance endogenous phospholipase activity, but the onset of these events would occur at a time associated with irreversible cell damage rather than during the period of reperfusion-induced arrhythmogenesis. However, the washout of lysophospholipids by reperfusion may prevent the resynthesis of membrane phospholipids

(reviewed by van der Vusse and Reneman, 1985), although the role of membrane repair in protection against reperfusion arrhythmias is speculative.

1.5.3 Catecholamines and Adrenergic Neural Activity

Clinical observations correlating increased sympathetic drive and elevated plasma catecholamine levels with the incidence of VF (Bertel *et al*, 1982) have been supported by experimental evidence of increased noradrenaline overflow in isolated rat hearts following regional and global ischaemia (Schomig *et al*, 1982; Abrahamsson *et al*, 1983). Ischaemia-induced release of myocardial noradrenaline is thought to occur by exocytosis of neuronal vesicles, although a reversal of the neuronal uptake 1 mechanism has been suggested (Schomig *et al*, 1984) after ischaemic periods of 10 min or more. Evidence associating sympathetic overactivity and elevated noradrenaline levels with arrhythmogenesis is equivocal; systemic administration of catecholamines has been shown to exacerbate or reduce arrhythmias during ischaemia (Harris *et al*, 1971), whilst chronic cardiac sympathetic denervation affords some protection (Fowlis *et al*, 1974). Chemical sympathectomy also reduces the incidence of ischaemic arrhythmias (Sheridan *et al*, 1980; Daugherty *et al*, 1986).

Attempts to reduce ischaemia-induced arrhythmias with beta-receptor blockade have produced conflicting results. Where antiarrhythmic protection is achieved, interpretation of data is complicated by

other non-specific actions of the beta-receptor antagonists used. Daugherty *et al* (1986) found both optical isomers of propranolol to be equipotent in suppressing arrhythmias, suggesting the involvement of a local anaesthetic (class I) antiarrhythmic mechanism. The results of a study by Curtis *et al* (1985) led the authors to suggest that beta-blockers may protect against arrhythmias by elevating the extracellular potassium concentration rather than by myocardial receptor blockade. Protection against reperfusion arrhythmias using propranolol (Sheridan *et al*, 1980), oxprenolol and timolol (Manning *et al*, 1983) has not been demonstrated, suggesting that activation of the adenylate cyclase system via beta-receptor stimulation is not involved in reperfusion arrhythmogenesis.

The alpha-adrenoceptor antagonists phentolamine and prazosin have been shown to be effective in reducing reperfusion arrhythmias (Sheridan *et al*, 1980). Corr and colleagues (1981) demonstrated enhanced alpha-receptor responsiveness in the cat myocardium and it has been argued (Corr and Witkowski, 1983) that increased alpha-receptor stimulation leads to changes in cardiac automaticity and may account for the increased idio- ventricular rate observed during reperfusion in cats (Sheridan *et al*, 1980). Alternative mechanisms have also been ascribed to the antiarrhythmic actions of phentolamine and prazosin. Electrophysiological studies on isolated heart tissues confirmed the local anaesthetic properties of phentolamine (Northover, 1983; Daugherty *et al*, 1986) and prazosin (Dukes and Vaughan-Williams, 1984). Phenoxybenzamine and trimazosin, alpha-receptor antagonists devoid of local anaesthetic

properties, did not significantly affect the occurrence of ischaemia-induced arrhythmias in the isolated rat heart (Daugherty *et al*, 1986).

The role of sympathetic activation in arrhythmogenesis therefore remains to be resolved. The relevance of elevated levels of myocardial catecholamines is subsequently difficult to determine. The evidence discussed above, together with the recent finding that the angiotensin converting enzyme inhibitors captopril, enalapril and ramiprilat, reduce noradrenaline overflow upon reperfusion of isolated rat hearts and protect against reperfusion arrhythmias (Linz *et al*, 1986; van Gilst *et al*, 1986), strongly support the view that catecholamines are arrhythmogenic under conditions of ischaemia and reperfusion. The possibility that noradrenaline is arrhythmogenic via a free radical-mediated mechanism is discussed later.

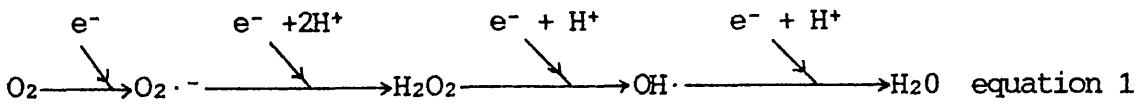
1.6 FREE RADICALS AND ARRHYTHMOGENESIS

Interest in free radical reactions has increased over the last 20 years to the extent that free radicals have been implicated as participants in a wide range of biological mechanisms including cancer (Oberley, 1982), ageing (Pryor, 1978), acute inflammation (Petrone *et al*, 1980), radiation damage (Misra and Fridovich, 1976) and ischaemia/reperfusion-induced damage (see below). Significant progress has been made in the understanding of mechanisms by which free radicals are generated and propagated, particularly those derived from molecular oxygen (O_2). The following considers the

importance of free radicals in ischaemia/reperfusion injury, with particular emphasis on the role of active oxygen metabolites in the generation of cardiac ventricular arrhythmias.

1.6.1 Definition,Types and Production of Oxygen Free Radicals

A free radical is defined as any species that independently exists with one or more unpaired outer electrons. This definition includes the hydrogen atom, most transition metals and O_2 itself, which has 2 unpaired electrons and so is referred to as a biradical. O_2 is a relatively slow reacting molecule. Its 2 outer electrons have parallel spins; this makes donation of another pair of electrons difficult, since most electron pairs have antiparallel spins. Addition of an electron pair would therefore require spin inversion of one O_2 electron, a process that takes a relatively long time compared to the lifetime of a collision complex. This phenomenon, known as "electron spin restriction", tends to result in one electron (univalent) additions to O_2. In mitochondria, O_2 is reduced tetravalently (4 electron addition) by virtue of enzyme systems that contain transition metals capable of accepting and donating single electrons (e.g. cytochrome oxidase). Even under normal aerobic conditions however, some univalent reduction occurs, giving rise to the active oxygen species superoxide ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) and finally water, as in equation 1;



equation 1

A condition that would favour the univalent pathway, and so the generation of oxygen free radicals, is the saturation of respiratory chain carriers situated on the inner mitochondrial membrane. This, in turn, would be brought about by an increased availability of reducing agents (Turrens *et al*, 1982).

1.6.2 Mechanisms of Free Radical Generation

Apart from the univalent reduction of oxygen outlined above, there are several other biological sources of $O_2 \cdot^-$. The first documented source was xanthine oxidase (McCord and Fridovich, 1968). Hypoxanthine and xanthine are metabolised in cells by virtue of the catalytic properties of the NAD^+-dependent dehydrogenase form of xanthine oxidase. Under certain conditions (extreme temperature and anoxia, and in the presence of proteolytic and sulphydryl oxidising agents), the dehydrogenase (D) form is converted to the oxidase (O) form (Parks and Granger, 1986). Xanthine oxidase acts as an electron acceptor in the catabolism of hypoxanthine and xanthine, using O_2 as the electron source. The removal of one electron from O_2 produces $O_2 \cdot^-$.

$O_2 \cdot^-$ may also be formed by the autoxidation of biological molecules including glyceraldehyde, cysteine and catecholamines. At physiological pH, the rates of oxidation of adrenaline and noradrenaline are slow, even in the presence of physiological

concentrations of transition metals (catalysts of free radical generation; see below)(Halliwell and Gutteridge, 1985). However, higher concentrations of metal catalysts such as iron (Fe^{3+} and Fe^{2+}) and copper (Cu^{2+}) accelerate this process and enhance free radical production. Of particular biological relevance is the oxidation of haemoglobin to oxyhaemoglobin. The former contains iron in its reduced (Fe^{2+}) form which, when attached to O_2, loses an electron resulting in the formation of an Fe^{3+}-$O_2\cdot^-$ complex. Such reactions occur in approximately 3% of human haemoglobin every day and so provide a constant flux of $O_2\cdot^-$ (Halliwell and Gutteridge, 1985).

$O_2\cdot^-$ is also used to the body's advantage. The bactericidal action of blood-borne neutrophils is thought to involve the chemotactic or physical stimulation of an NADPH oxidase complex in the plasma membrane of the phagocytic cell (Babior, 1978). Activation of this complex triggers the uptake of O_2 by the neutrophil, a process known as the "respiratory burst". NADPH is used as an electron source for the reduction of O_2 to form $O_2\cdot^-$ (equation 2);

$$NADPH + 2O_2 \longrightarrow NADP^+ + H^+ + 2O_2\cdot^- \qquad \text{equation 2}$$

In the aqueous interior of cells and cell organelles, $O_2\cdot^-$ undergoes spontaneous "dismutation" (equation 3);

$$2O_2\cdot^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{equation 3}$$

The rate of this reaction is slow (K_m<0.3 $M^{-1}s^{-1}$), but is

accelerated by acidic conditions and in the presence of enzyme catalysts, antioxidants and transition metals (Halliwell and Gutteridge, 1985). Equation 1 shows that any system which generates $O_2\cdot^-$ must also produce H_2O_2, unless $O_2\cdot^-$ is removed from that system. H_2O_2 has no unpaired electrons and so is not strictly a free radical, although it is often classed as such because of its involvement in the production of $OH\cdot$ from $O_2\cdot^-$.

Homolytic fission of the O-O bond in H_2O_2 produces two $OH\cdot$ radicals, a process which is achieved by heat or ionising radiation (Halliwell and Gutteridge, 1984). The participation of transition metals in $OH\cdot$ formation is more physiologically relevant. In 1894, Fenton first described a role for iron in a reaction known as the Fenton reaction, as described by Halliwell and Gutteridge (1984) (equation 4);

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^- \qquad \text{equation 4}$$

The reaction between $O_2\cdot^-$ and H_2O_2 was also postulated as a mechanism of $OH\cdot$ production (Haber and Weiss, 1934) and is known as the Haber-Weiss reaction (equation 5);

$$O_2\cdot^- + H_2O_2 \longrightarrow O_2 + OH^- + OH\cdot \qquad \text{equation 5}$$

Although the rate constant for the above reaction is vitually zero in aqueous solution (Halliwell and Gutteridge, 1984), the mechanism is plausible in the presence of transition metals such as iron. This "iron-catalysed Haber-Weiss reaction" involves the reduction of Fe^{3+} ions by $O_2\cdot^-$ followed by the Fenton reaction, and is shown

in equations 6 and 7;

$$Fe^{3+} + O_2\cdot^- \longrightarrow Fe^{2+} + O_2 \qquad \text{equation 6}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^- \qquad \text{equation 7}$$

The importance of transition metals in oxygen radical generation is therefore apparent and it is not surprising that the body uses iron-binding proteins (transferrin, lactoferrin and ferritin) to keep the non-protein-bound (free) iron pool as small as possible (Halliwell and Gutteridge, 1984).

1.6.3 Oxygen Radical-mediated Damage and Cellular Defence Mechanisms

$O_2\cdot^-$ itself has the capacity to attack the biologically ubiquitous carbonyl (C = O) group, and so could be extremely damaging to membrane phospholipids. However, as was shown by equation 3, $O_2\cdot^-$ undergoes spontaneous dismutation, the rate of which is increased by the catalytic action of endogenous enzymes called superoxide dismutases (SOD's) (McCord and Fridovich, 1968). SOD's are proteins containing metal groups. In mammals, two types of SOD are thought to be important in $O_2\cdot^-$ dismutation; Cu-Zn SOD and Mn SOD (Steinman, 1982). The former is mainly a cytosolic enzyme but has also been located in lysosomes and between the inner and outer mitochondrial membranes. Mn SOD is located in the mitochondrial matrix. The active sites of Cu-Zn SOD and Mn SOD, Cu^{2+} and Mn^{3+} ions respectively, undergo the reaction sequence shown in equations 8 and 9;

$$\text{Enzyme-}M^{n+} + O_2\cdot^- \longrightarrow \text{Enzyme-}M^{(n-1)+} + O_2 \qquad \text{equation 8}$$

$$\text{Enzyme-}M^{(n-1)+} + O_2\cdot^- + 2H^+ \longrightarrow \text{Enzyme-}M^{n+} + H_2O_2 \qquad \text{equation 9}$$

where M represents the metal atom

SOD's are specific for $O_2\cdot^-$ only, and the rate of the catalysed dismutation reaction is of the order of $5x10^5\ M^{-1}s^{-1}$. It is therefore unlikely that $O_2\cdot^-$ is responsible for much oxygen radical-mediated damage to biological systems. The product of $O_2\cdot^-$ dismutation, H_2O_2, is a weak oxidizing agent capable of oxidizing thiol (-SH) groups and in doing so may inactivate thiol-containing enzymes (Halliwell and Gutteridge, 1985). However, most aerobic cells possess catalases, located mainly in subcellular organelles called peroxisomes, which catalyse the reduction of H_2O_2 to water (equation 10);

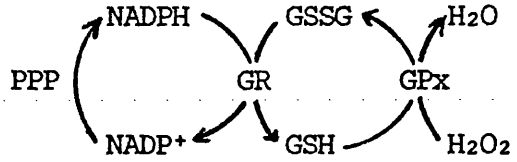
$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad \text{equation 10}$$

Mammals also possess glutathione peroxidase, an enzyme which utilises the low molecular weight thiol compound glutathione (present in cells in mM quantities as the reduced form, GSH) to reduce H_2O_2 (equation 11);

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O \qquad \text{equation 11}$$

This enzyme system is thought to be important in cellular locations, such as mitochondria, where catalase activity is either very low or absent (Kosower and Kosower, 1978). When both catalase

and glutathione peroxidase systems are available, the enzymes are thought to "co-operate"; as the concentration of H_2O_2 increases, catalase activity increases to take-over from glutathione peroxidase in removing H_2O_2 (Halliwell and Gutteridge, 1985). The importance of co-operativity is apparent when the GSH regeneration pathway is considered;



scheme 1

As was seen in equation 11, the reduction of H_2O_2 by glutathione peroxidase (GPx) at the expense of GSH produces oxidised (disulphide) glutathione, GSSG. The regeneration of GSH from GSSG (scheme 1 above) is achieved using the enzyme glutathione reductase (GR), which utilises reduced nicotine adenine dinucleotide phosphate (NADPH) produced by the pentose phosphate pathway (PPP). The rate limiting step of the PPP is the dehydrogenation of glucose-6-phosphate to 6-phosphogluconate, a reaction catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH). If the rate of H_2O_2 production exceeds the capacity of G-6-PDH to produce NADPH, then the GSH/GSSG ratio falls (Kosower and Kosower, 1978).

The endogenous mechanisms for "scavenging" $O_2\cdot^-$ and H_2O_2 would, under normal physiological conditions, be expected to protect cells against oxygen radical damage. However, if these mechanisms are perturbed or overwhelmed by excessive free radical production, $OH\cdot$ is produced by the transition metal-catalysed Fenton and

Haber-Weiss reactions (equations 6 and 7). Since OH· is the most reactive biologically-produced chemical species known (Halliwell and Gutteridge, 1985), the production of OH· is potentially lethal to cells. OH· undergoes 3 main types of reaction (Willson, 1978); hydrogen abstraction, addition and electron transfer. In cells, OH· removes hydrogen from the lipids of membranes to produce lipid peroxides, as illustrated below;

$$\text{Lipid-H} + \text{OH}\cdot \longrightarrow \text{Lipid}\cdot + H_2O \qquad \text{equation 12}$$

$$\text{Lipid}\cdot + O_2 \longrightarrow \text{Lipid-O-O}\cdot \qquad \text{equation 13}$$

$$\text{Lipid-O-O}\cdot + \text{Lipid-H} \longrightarrow \text{Lipid-O-O-H} + \text{Lipid}\cdot \qquad \text{equation 14}$$

Initiation (equation 12) of the lipid peroxidation sequence, the abstraction of hydrogen by OH·, is proceeded by the formation of lipid peroxy radicals (Lipid-O-O·, equation 13) by the reaction of oxygen with a conjugated diene (i.e. Lipid· following electron rearrangement). Peroxy radicals combine with hydrogen atoms on other lipid molecules, resulting in the formation of lipid hydroperoxides (Lipid-O-OH, equation 14). The lipid peroxidation process has been extensively reviewed by Halliwell and Gutteridge (1985, chapter 4).

Peroxidation of membrane lipids perturbs the structure and function of membranes; changes in fluidity, surface charge, electrical stability and ion permeability have all been demonstrated (see Kako, 1985, for review). Membrane-bound enzyme systems, such as sodium-potassium ATPase, calcium ATPase and adenylate cyclase, are similarly disturbed (Stekhoven and Bonting, 1981). Protection

against such damage is therefore essential for normal cellular function and as a consequence, several other defence mechanisms exist in addition to those previously considered. Vitamin C (ascorbic acid) and vitamin E (alpha-tocopherol) are lipid soluble molecules located in biological membranes which react with OH· and lipid peroxy radicals to produce other less reactive radical species (Freeman and Crapo, 1982). Similarly, GSH can react directly with OH· (Halliwell and Gutteridge, 1985).

Under normal physiological conditions, it would appear that cells possess adequate enzymatic and antioxidant defence mechanisms to protect against the deleterious effects of active oxygen metabolites. Many authors (Meerson *et al*, 1982; Hess and Manson, 1984; Otani *et al*, 1984; McCord, 1985; van der Vusse and Reneman, 1985; Woodward and Zakaria, 1985; Bernier *et al*, 1986; Werns *et al*, 1986) have proposed that such mechanisms are overwhelmed by accentuated radical production in the setting of myocardial reperfusion. The following paragraphs will therefore consider the hypothesis that oxygen free radicals are generated during ischaemia and/or reperfusion of the heart and contribute to the pathogenesis of life-threatening cardiac arrhythmias.

1.6.4 Free Radical Generation in the Ischaemic/Reperfused Heart

Several changes that occur during ischaemia/reperfusion have the potential to increase the extent of free radical formation (table 1.6.4). In ischaemic cells, the decrease in O_2 available for the

1: ACCUMULATION OF REDUCED METABOLITES
2: MITOCHONDRIAL DAMAGE
3: CALCIUM OVERLOAD
4: ARACHIDONIC ACID METABOLISM
5: AUTOXIDATION OF CATECHOLAMINES
6: XANTHINE OXIDASE
7: NEUTROPHIL ACTIVATION

Table 1.6.4:

Putative sources of oxygen free radicals in the ischaemic reperfused heart.

tetravalent respiratory pathway inhibits the terminal component of the respiratory chain, cytochrome oxidase (Meerson *et al*, 1982). Any available O_2 dissolved in the lipid matrix of cell membranes would therefore tend to be utilised by the univalent pathway. Reduced metabolites such as NADH accumulate under ischaemic/hypoxic conditions (Opie, 1978; Meerson *et al*, 1982) as a result of an initial increase, followed by cessation, of anaerobic glycolysis and so provide a source of electrons for univalent O_2 reduction. The reintroduction of O_2 to this system (during reperfusion, for example) would provide an abundant supply of substrate for the univalent pathway (particularly if mitochondria are damaged, see below) and may enhance the formation of oxygen radicals. Inhibition of cytochrome oxidase with cyanide has indeed been shown to enhance $O_2\cdot^-$ formation (Chance *et al*, 1977; Turrens *et al*, 1982).

Since mitochondria are the major site of cellular oxidation reactions, it seems reasonable to suppose that they are also the main site for the generation of oxygen radicals. Indeed, Guarnieri *et al* (1985) demonstrated that sub-mitochondrial particles from ischaemic rabbit heart produce more $O_2\cdot^-$ than those from normal heart. In an earlier publication, Guarnieri and co-workers (1983) exposed rat heart mitochondrial preparations to $O_2\cdot^-$ generated by the xanthine/xanthine oxidase system and observed a reduced ability to utilise anaerobic glycolytic substrates. The authors proposed that inhibition of this energy production pathway would lead to univalent O_2 reduction and consequently the production of more oxygen radicals. Under conditions of saturated mitochondrial (tetravalent) respiratory function, hyperoxia or an increase in O_2

tension leads to univalent O_2 reduction and free radical generation (Freeman and Crapo, 1981; Turrens *et al*, 1982). During no-flow ischaemia, the initial increase in anaerobic glycolysis is inhibited by the accumulation of lactate and pyruvate in the ischaemic tissue (Neely and Feuvray, 1981) and the ensuing decrease in ATP synthesis inevitably slows-down energy-dependent processes such as those involved in the maintenance of myocardial ion gradients. Increases in mitochondrial potassium efflux (Masini *et al*, 1984) and mitochondrial calcium overload (Hess and Manson, 1984; Nayler, 1981) have been described under ischaemic conditions. At present, a definitive causal effect relationship between free radical generation, mitochondrial damage and mitochondrial calcium overload during ischaemia and reperfusion is lacking. Whilst free radical-induced mitochondrial damage may predispose to mitochondrial calcium overload, it is equally possible that mitochondrial calcium overload enhances free radical production by inhibiting mitochondrial oxidative phosphorylation (Braughler *et al*, 1985). Despite this lack of clarity, the fact remains that reoxygenation of anoxic myocardium is associated with structural (Hearse *et al*, 1985) and functional (Opie, 1978) damage to mitochondria, which in conjunction with the protective effect of free radical scavengers on mitochondrial function (Guarnieri *et al*, 1978), strongly implicates mitochondria as a prime source of oxygen free radicals during myocardial reperfusion.

The release and accumulation of prostanoids during myocardial ischaemia was considered earlier (section 1.5.1). Whilst the arrhythmogenic role of prostanoids is not fully understood, the

ischaemia-associated metabolism of arachidonic acid to prostaglandins and leucotrienes (Coker *et al*, 1981) generates electrons capable of initiating free radical formation (Rowe *et al*, 1983). Indeed, Khuel and colleagues (1980) observed the production of a powerful oxidant, probably $OH\cdot$, during the conversion of prostaglandin G_2 to prostaglandin H_2. Calcium-activated phospholipases stimulate arachidonic acid metabolism (Blackwell and Flower, 1983) and it has been suggested (van der Vusse *et al*, 1982) that reperfusion-induced calcium overload may be related to oxygen radical formation by this mechanism.

Elevated levels of catecholamines, particularly noradrenaline, during ischaemia (section 1.5.3) may provide an additional source of oxygen radicals during reperfusion. Catecholamine autoxidation makes available electrons for the reduction of O_2. Evidence in support of such a mechanism was presented by Singal and colleagues (1983), who were able to protect against isoprenaline-induced myocardial damage in vitamin E-deficient rats by supplying a dietary source of the vitamin.

The xanthine/xanthine oxidase system is frequently used for the experimental generation of oxygen free radicals. This system was first characterised by McCord and Fridovich (1968). The presence of endogenous xanthine oxidase has been demonstrated in many tissues (McCord, 1984), predominantly as the dehydrogenase form (Schoutsen *et al*, 1983). Xanthine dehydrogenase catalyses the metabolism of hypoxanthine and xanthine to uric acid using NAD^+ as an electron

acceptor and so does not produce free radicals (Chambers *et al*, 1985). Evidence suggests that under certain pathological conditions such as myocardial ischaemia (Chambers *et al*, 1985; Granger *et al*, 1986), the dehydrogenase is converted to the oxidase by proteolysis (McCord, 1985) and/or sulphydryl oxidation (Waud and Rajagopalan, 1976). Xanthine oxidase catalyses the same reactions as the dehydrogenase, the important difference being its use of O_2 as an electron acceptor. Therefore, xanthine oxidase-catalysed reactions produce $O_2\cdot^-$. That xanthine oxidase is a physiological source of oxygen radicals is now generally accepted. Dehydrogenase to oxidase conversion during myocardial ischaemia, followed by the introduction of O_2 during reperfusion, provide ideal conditions for xanthine oxidase-derived oxygen radical production. The involvement of endogenous xanthine oxidase in reperfusion damage is considered further in chapter 6.

The contribution of the $O_2\cdot^-$-producing action of phagocytic cells to ischaemic damage has also been proposed (Engler *et al*, 1983; Romson *et al*, 1983; Mullane *et al*, 1984). Following the onset of myocardial ischaemia, the myocardium is invaded by activated neutrophils as part of the inflammatory process (Romson *et al*, 1983; Mullane *et al*, 1984). The $O_2\cdot^-$ released by these cells may contribute to myocytolysis although the duration of the migration process is critical to such a role. A 17-fold increase in the neutrophil content of the myocardium has been observed 24 hours after infarction (Romson *et al*, 1983), whereas Engler and colleagues (1983) could not demonstrate any neutrophil migration following 5 hours of ischaemia. The role of neutrophils in

mediating oxygen radical-derived damage during early ischaemia is therefore unresolved and evidence for such a role in reperfusion arrhythmogenesis is lacking (Woodward and Manning, 1987). The ability to induce reperfusion arrhythmias in isolated hearts, in which there are likely to be very few if any neutrophils present, would further suggest that neutrophils are not a major factor in the initiation of these arrhythmias.

In summary, many physiological mechanisms exist with the potential to generate damaging free radicals. Circumstantial evidence suggests that conditions of ischaemia and reperfusion are conducive to the involvement of such mechanisms and their contribution to reperfusion-induced arrhythmogenesis should be a prime consideration in studies of ischaemic heart disease and sudden cardiac death.

1.7 OBJECTIVES OF THE PRESENT STUDY

The following study was aimed at evaluating the working hypothesis that reperfusion arrhythmias are associated with mechanisms involving the generation of oxygen free radicals. The aims of individual experiments are detailed in the appropriate chapters, but can be integrated into the following main objectives;

(1) to confirm that reperfusion of regionally ischaemic isolated rat hearts evokes ventricular arrhythmias;
(2) to demonstrate that reperfusion arrhythmias are accompanied by the production of oxygen free radicals;

(3) to demonstrate that free radical scavengers prevent or reduce the development of ventricular arrhythmias;

(4) to investigate the importance of potassium ion fluxes to arrhythmogenesis and the effect of free radical generation on potassium efflux from hearts;

(5) to investigate the role of putative free radical generating systems in reperfusion arrhythmogenesis.

CHAPTER 2

METHODOLOGY

2.1 THE ISOLATED RAT HEART PREPARATION. REGIONAL ISCHAEMIA AND REPERFUSION

Hearts from male wistar rats (200g to 300g, University of Bath strain) were canulated via the aorta and perfused retrogradely at a constant flow of 10ml/min with a bicarbonate buffer of the following composition (mM):

NaCl,118;KCl,4.7;KH_2PO_4,1.2;$MgSO_4$,1.2;$NaHCO_3$,25;$CaCl_2$,1.2;D-glucose,11.1.

The perfusion fluid was gassed with 95%O_2/5%CO_2 and was of pH 7.4 at 37°C. During preparation of the perfusion fluid, the precipitation of calcium was carefully avoided and removal of particulate matter from all solutions was facilitated by on-line filtration. Immediately after the heart was mounted, a loose suture was placed around the left anterior descending coronary artery close to its origin. Both ends of the ligature were passed through a short polythene tube to form a snare. Regional ischaemia could be induced by pulling the suture whilst pressing the tube against the myocardial surface, where it was held in place with a small clip. A sustained increase in perfusion pressure indicated successful coronary artery ligation (CAL). Reperfusion (REP) could be initiated by unclipping and removing the tube; a decrease in perfusion pressure indicated successful reperfusion. Further evidence of successful ligation was obtained at the end of each experiment, when hearts were perfused with disulphine blue and the ligation procedure repeated. Electrocardiogram (ECG) electrodes were placed on the epicardial surface of the right atrium and left ventricle and ECG's were monitored on a Narco Trace (Narco Biosystems, USA) oscilloscope. Tension developed from a diastolic

load of 2g was recorded via a Devices UF1 isometric transducer attached by cotton thread and fine nickel hooks to the apex of the left ventricle. The tension recording was also used to trigger a Devices 4521 rate meter in order to monitor heart rate. Perfusion pressure was monitored using a Bell Howell 4-442 pressure transducer. Each parameter was recorded on a Gould 2400S 4 channel recorder. Developed tension was recorded simultaneous with ECG's on a Devices MX216 2 channel recorder at a fast chart speed.

2.1.1 Drug Perfusion Protocol

Hearts were allowed an initial equilibration period of 15 min, during which standard perfusion medium was used. For the following 5 min, the composition of the perfusion fluid was changed by switching to a reservoir containing bicarbonate buffer with a potassium concentration of 3.2mM in order to enhance the development of reperfusion-induced arrhythmias (Woodward and Zakaria, 1983). During this period, drug-free control values for contractility, heart rate and perfusion pressure were recorded. Hearts were then perfused with the drug under investigation (solubilised in low potassium buffer) for 5 min, following which CAL was induced for a further 10 min. The effects of reperfusion were then studied for 3 min, during which time epicardial ECG's were recorded. Control electrical activity was recorded 5 sec prior to reperfusion.

2.1.2 Hypoxia Protocol

Hypoxia was induced either 2 min before CAL or 2 min before reperfusion. Since no drug was being used, hearts were perfused with low potassium perfusate throughout the experiment after the initial equilibration period. Perfusion fluid was rendered hypoxic by gassing with 95% N_2/5% CO_2.

2.1.3 Perfusion with "Arrhythmogenic" Solution

In experiments designed to induce arrhythmias using an "arrhythmogenic" solution, hearts were perfused with a buffer in which the concentrations of KCl and $MgSO_4$ were reduced to 1.2mM and zero respectively, and the $CaCl_2$ concentration was increased to 4.9mM. Arrhythmogenic perfusion commenced 5 min after perfusion with low potassium buffer. Ventricular fibrillation (VF; see section 2.3) occurred 220±11 sec after arrhythmogenic perfusion began (n=5).

2.2 DETERMINATION OF POTASSIUM EFFLUX USING THE RADIOACTIVE TRACER ION RUBIDIUM - 86 ($^{86}Rb^+$)

2.2.1 Experimental Protocol

Hearts were isolated and perfused as previously described (section 2.1) . Following a 5 min equilibration period, hearts were loaded with 0.2 μCi/ml $^{86}Rb^+$ in standard perfusion medium (5.9mM K^+) for 10 min. The next 30 min comprised a washout period under standard

perfusion conditions, after which hearts were perfused with low potassium (3.2mM K^+) buffer. The remainder of the experiment was as described in section 2.1. Radioactive perfusate samples were collected every 2 min when required and every 30s following reperfusion. At the end of the experiment, ischaemic and non-ischaemic areas of the heart were identified (section 2.1), each was blotted dry, weighed and minced with scissors. To each tissue sample, 5 ml 1M KOH was added and the samples were allowed to digest for 48 hours.

2.2.2 Treatment of Samples

To each tissue suspension, 5 ml 1M HCl were added to neutralise the sample and prevent chemiluminescence interference (Durbin and Jenkinson, 1961) during subsequent scintillation counting. Aliquots of 1 ml were taken from perfusate or neutralised tissue samples, to which 4 ml Optiphase "Safe" scintillation cocktail were added. After thorough mixing, samples were analysed in an LKB 1215 liquid scintillation counter (Durbin and Jenkinson, 1961).

2.2.3 Calculation of the Efflux Rate Coefficient (erc)

The efflux rate coefficient (erc) at time t was calculated according to equation 15;

$$\text{erc } (\text{min}^{-1}) = \frac{\text{perfusate count}}{\text{total count} \times \text{collection time (min)}} \qquad \text{equation 15}$$

where the total count is the tissue count plus the sum of the perfusate counts up to time t.

In order to speed-up calculations, a computer programme was written (see Appendix 1).

2.3 EVALUATION OF ARRHYTHMIAS

Arrhythmic activity was assessed by counting the number of premature ventricular contractions (PVC's), including those defined as ventricular tachycardia (VT; see below), and by recording the incidences, onset and duration of VT and ventricular fibrillation (VF). Isolated PVC's were defined as discrete QRS complexes and were inevitably followed by a compensatory pause in electrical activity and an increase in the amplitude of the resultant contraction. VT was classified as 5 or more consecutive PVC's and was accompanied by a reduction in contraction amplitude. Bigeminal rhythms, when occuring 3 or more times consecutively, were also classified as VT. When discrete QRS complexes were no longer clearly identifiable and electrical signals exhibited chaotic activity with regard to amplitude, hearts were deemed to be undergoing VF. In such cases, contractility was greatly reduced or, as was usually observed, absent. An increase in resting tension occurred during episodes of VF.

2.4 BIOCHEMICAL METHODS

2.4.1 Protein Estimation

All protein determinations were carried out using the simple and rapid Coomassie blue dye-binding method of Bradford (1976). Calibrations were made using bovine serum albumin (BSA) as standard protein.

2.4.2 Ferricytochrome c Reduction

Hearts were perfused with low potassium (3.2mM) buffer containing $5x10^{-5}$M ferricytochrome c (FeCytC). Perfusate samples were collected and their light absorbances measured at 550nm wavelength in a Pye Unicam PU8605 spectrophotometer (Goldstein *et al*, 1975).

The reduction of FeCytC by xanthine oxidase in the presence of xanthine, and investigations with agents which might affect this process, were carried out as below. The procedure described was modified from the method of Horecker and Heppel (1949). To 1ml of reagent mixture (comprising $1x10^{-5}$M FeCytC, $5x10^{-5}$M xanthine, $1x10^{-4}$M disodium EDTA and $5x10^{-2}$M tripotassium phosphate; pH 7.4 at 25°C) in a cuvette, 0.05ml water (or the agent under investigation) was added. After mixing, the reduction of FeCytC was initiated by the addition of 0.05ml (0.65U) xanthine oxidase and monitored in a Cecil CE595 spectrophotometer coupled to a Cecil CE500 chart recorder at a wavelength of 550nm. The rate of the reaction was calculated qualitatively as the change in absorbance units per unit

time.

2.4.3 The Determination of Lactate and Lactate Dehydrogenase (LDH)

Lactate was determined by a method similar to that of Hohorst (1965). To 1ml of buffer ($5x10^{-1}$M glycine, $2x10^{-1}$M hydrazinium sulphate; pH adjusted to 9.5 with 2M NaOH at 25°C) in a quartz cuvette, 0.05ml of $5x10^{-2}$M NAD and 0.1ml of sample solution were added. After mixing, the absorbance of the solution was read at 340nm wavelength in a Pye Unicam PU8605 spectrophotometer with a deuterium light supply. 0.005ml (10U/ml) LDH was subsequently added, the solution mixed and allowed to stand at room temperature for 30 min before a second absorbance reading was taken. The concentration of lactate in each sample was calculated using equation 16;

$$\mu\text{moles/ml lactate} = \frac{dE \times \text{assay volume} \times 10}{e} \qquad \text{equation 16}$$

where: dE is the difference between the 2nd and 1st absorbance readings; e is the mM extinction coefficient of NADH at 340nm (= 6.22).

The assay for LDH was based on the same oxidation-reduction reaction involved in lactate determination (equation 17);

$$\text{PYRUVATE} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{LACTATE} + \text{NAD}^+ \qquad \text{equation 17}$$

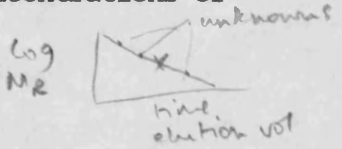
LDH could therefore be determined by the reduction of pyruvate or by the oxidation of lactate. The assay procedure described below used the latter principle. 1ml of $5x10^{-1}$M sodium phosphate buffer (pH 7.6), 0.4ml of $5x10^{-1}$M L-lactate and 0.3ml of $1.4x10^{-2}$M NAD solution were mixed in a quartz cuvette. A 1ml aliquot of sample solution was added and after mixing, the absorbance of the solution was read at 340nm wavelength at 30s intervals for 8 min in a Pye Unicam PU8605 spectrophotometer with a deuterium light source. Since 1 unit of enzyme oxidises 1µmole of NAD^+ per min (Eichner, 1982), LDH activity could be calculated from the slopes of the resultant linear plots.

2.4.4 Removal of Ammonium Sulphate and Sodium Salicylate from the Commercial Preparation of Xanthine Oxidase

Although the effect on hearts of laboratory preparations of the vehicle for xanthine oxidase was studied, it seemed desirable to test the effects of the enzyme in the absence of the vehicle. Should no apparent differences in response be observed, the time-consuming removal of the vehicle could be avoided. To this end, 1ml of xanthine oxidase was dialysed using Medicell 18/32" dialysis tubing in 10 litres of 0.9% saline at 4°C for 24 hr in the dark. Dialysis tubing was prewashed in double distilled, deionised water to remove metal ion and other contaminants which might have interfered with the enzyme's activity. Following dialysis, samples of enzyme were tested for activity and protein content as

previously described.

A second technique for removal of the vehicle, gel filtration, was also used. Sephadex G-25 microspheres were pre-swollen and suspended in $5x10^{-2}M$ Tris/HCl buffer, pH 7.6 at 25°C, and a 25ml column was prepared. Commercial xanthine oxidase (1ml) was added to the column and eluted with the Tris/HCl buffer. Fractions of 1ml were collected and underwent qualitative protein assay by measuring their absorbances at 280nm. To test for the presence of ammonium sulphate, each fraction was subjected to a barium sulphate precipitation test. To 0.1ml of sample, 1ml of saturated barium chloride was added. The presence of sulphate was indicated by the appearance of a white precipitate of barium sulphate. Preliminary studies showed this method to be sensitive to concentrations of sulphate exceeding $10^{-4}M$.



2.5 CHEMILUMINESCENCE MEASUREMENTS OF XANTHINE OXIDASE AND XANTHINE DEHYDROGENASE IN CARDIOVASCULAR CELLS

2.5.1 Cell Culture

Neonatal cardiac myocytes in primary culture were obtained according to the method of Higgins and co-workers (1979). They were purified to approximately 95% by differential adherence (von Tscharner and Bailey, 1983). Monolayers were maintained in potassium-free salt solution (NaCl, 116mM; $MgSO_4$, 1mM; $CaCl_2$, 1.8mM; d-glucose, 5.6mM) containing 2% Hams F10 (x10) medium, 10% HEPES, 10% foetal calf serum (FCS), 2.5µg/ml gentamycin, 2mM

l-glutamine and 380µg/ml penicillin. Fibroblasts were grown from the same primary cell preparations. They were allowed to proliferate beyond confluence until, after 5 passages in the above medium, 100% fibroblasts were identified morphologically.

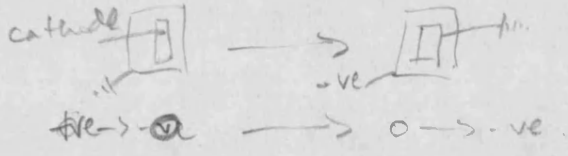
Rat aortic vascular smooth muscle (vsm) cells were obtained from PLHS Centre of Applied Microbiology and Research, Porton Down (U.K.) as a pure cell line (A10) stored in liquid nitrogen. They were cultured for up to 5 passages in Dulbecco's Modified Eagles Medium (DMEM) containing 10% FCS, 2mM l-glutamine and 10µg/ml gentamycin.

Adult rat myocytes were prepared from male Wistar rats (260-300g; Alderley Park Strain, I.C.I., U.K.) using the method of Powell and co-workers (1980). Cell yield was typically $3x10^5$/ml/heart of 50% to 80% striated, rod-shaped cells. Cells were used immediately after the assessment of density, morphology and viability (trypan blue exclusion).

Endothelial cells were prepared from human umbilical vein as described by Gimbrone *et al* (1974). They were passaged for up to 2 weeks in Medium 199 containing 10% sterilised fresh human serum, 10% FCS, 50ng/ml fibroblast growth factor, 2mM l-glutamine and 5µg/ml gentamycin.

All cells were maintained at 37°C in 150ml Costar flasks in an incubator (LEEC mark II). Neonatal and adult myocytes were maintained in an air-equilibrated atmosphere. All other cells were

equilibrated with 5% CO_2. Cells were grown to confluency in 9cm² single culture dishes (Costar).



2.5.2 Chemiluminescence Detection

Photons emitted by chemiluminescence were detected using a 52mm EMI 9635QA phototube operating at 1050 volts in the negative ground configuration to reduce corona effects. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), the agent used to enhance the signal, has an emission maximum at 425nm wavelength (Miura and Ogiso, 1985). The phototube used was sensitive to wavelengths below 570nm. The phototube was cooled to 4°C in a FACT 50 mark III cooler, with a quartz double window separating it from the sample compartment below. The distance between the phototube and the cells was 50mm, the minimum allowing a rotational access of the culture plate to the light-tight sample compartment. Phototube emission was fed via a very short (10cm) co-axial lead to an 1121A amplifier-discriminator and then to an 1109 photon counter (both from E.G. and G.Princeton Applied Research). The response time of the detection system, typically 3ns, compared well with the rise time of the phototube, 10ns. Discrimination thresholds were set daily by observing the pulse height analyser output of the 1121A on an oscilloscope. Data were collected in the single window mode at ambient temperature (19°C).

2.5.3 Protocol for Measurement of Xanthine Oxidase/Dehydrogenase in Cell Preparations

Cell monolayers were introduced into the sample compartment in 2ml of the appropriate medium containing $4x10^{-4}M$ xanthine and 40µl of 2mg/ml luminol. Cells were then lysed by the addition of 40µl of 1% (v/v) Triton-X100 in double distilled, deionised water and the chemiluminescence immediately measured for 10 consecutive periods of 10s. 40µl of $5x10^{-3}M$ 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in phosphate buffered saline was then added to oxidise any dehydrogenase present to the oxidase (Waud and Rajagopalan, 1976) and the measurements were repeated. Finally, 5mU (40µl) of xanthine oxidase was added as a positive control. Measurements on adult myocytes were performed in suspension, the cells being resuspended in medium containing xanthine after harvesting at 300rpm for 1 min. Experiments on endothelial cells were repeated in the presence of $10^{-6}M$ oxypurinol, an inhibitor of xanthine oxidase.

All experiments were performed in triplicate unless otherwise stated. Enzyme activities were expressed per unit of cell protein following protein determination as previously described. Cell samples were randomly assigned prior to each chemiluminescence experiment.

The antioxidant properties of each medium were compared by challenging samples with 15mU xanthine oxidase in the presence and absence of 10U/ml superoxide dismutase (SOD), and in separate experiments by measurement of the rate of reduction of FeCytC

induced by xanthine oxidase in the presence and absence of each medium (see section 2.4.2).

2.6 STATISTICAL ANALYSIS

Results are expressed as the mean ± the standard error of the mean unless otherwise specified. Changes in haemodynamic parameters were compared to pre-treatment values using Student's paired t-test. Differences from control values of the number of PVC's and the onset and duration times of both VT and VF were analysed using the Mann-Whitney U test. The incidences of VT and VF in control and treated groups were compared by Fischer's Exact test. Regression lines were fitted by least squares analysis where appropriate. The efflux of $^{86}Rb^{+}$ following reperfusion was analysed by plotting erc against time and measuring the area bounded by the curve for the first 3 min of reperfusion and by the pre-reperfusion baseline (calculated as the mean of the pre-reperfusion values). Mean ± sem values of area (in arbitrary units, au) and peak erc were calculated for each experimental group, and comparisons between control and treated group means were made using the Mann-Whitney U test.

Statistical significance was assumed for all tests when the probability of the difference occurring by chance was equal to or less than 5% (i.e. $p \leq 0.05$).

2.7 THE HOUSING AND FEEDING OF ANIMALS

All rats were housed 10 to a cage and were constantly supplied with standard rat chow and tap water. Animal house lighting was set for an 8 hr light-8hr dark cycle.

2.8 EXCLUSION CRITERIA FOR HEART PREPARATIONS

As previously stated (section 2.1), isolated perfused heart preparations were allowed an initial equilibration period of usually 15 min. If, at the end of this period, heart rate,perfusion pressure and contractility were not stable, the experiment was terminated. Although excluded hearts were not recorded, it should be noted that such incidences rarely occurred.

2.9 MATERIALS

Chemical	Source
Allopurinol	Sigma
Bovine serum albumin (fraction V)	Sigma
Catalase	Sigma
Coomassie brilliant blue G-250	Sigma
Cytochrome c (type III from horse heart)	Sigma
Desferrioxamine mesylate	Ciba
Diethyldithiocarbamate (DDC)	Sigma
Disulphine blue	Sigma
Dithiobisnitrobenzoic acid (DTNB)	Sigma
Dithiothreitol (DTT)	Sigma
Dulbecco's Modified Eagles Medium (DMEM)	Gibco
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Fibroblast growth factor (FGF)	Gibco
Foetal calf serum (FCS)	Gibco
Gentamycin	Sigma
L-glutamine	Sigma
Glutathione (reduced and oxidised; GSH and GSSG)	Sigma
Glycine	Sigma
Hams F10 (x10) medium	Gibco
HEPES	Sigma
Hydralazine HCl	Sigma
Hydrazinium sulphate	Sigma
6-hydroxydopamine (6-OHDA)	Sigma
L-lactate	Fisons

Lactate dehydrogenase (LDH; from rabbit muscle)	BCL
Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione)	Sigma
Mannitol	BDH
Nicotinamide adenine dinucleotide (NAD)	BCL
Optiphase "Safe"	LKB
Oxypurinol	Sigma
D-penicillamine (PSH)	Sigma
Penicillin	Gibco
Rubidium-86 chloride ($^{86}Rb^{+}$)	Amersham
Sephadex G-25 (20-50μm swollen bead size)	Sigma
Sodium nitroprusside	Sigma
Sodium salicylate	Sigma
Superoxide dismutase (SOD, E.C.1.15.1.1; from bovine erythrocytes)	Sigma
Triton-X100	Sigma
Tyramine HCl	Sigma
Xanthine	Sigma
Xanthine oxidase (E.C.1.2.3.2; type III from buttermilk)	Sigma

N.B. Abbreviated drug names are shown in brackets unless defined in the text.

CHAPTER 3

3.1 INTRODUCTION: THE ISOLATED RAT HEART AS AN EXPERIMENTAL MODEL OF MYOCARDIAL ISCHAEMIA/REPERFUSION

As with all experimental models, no species is ideal for the comparison of physiological and pharmacological responses in Man. However, small animals such as the rat are regularly used in studies where large experimental numbers are required. The low collateral flow observed in the rat heart allows induction of a rapid and almost complete ischaemia following coronary artery occlusion (Schaper, 1984), a situation analagous to that which may be expected in a young human heart undergoing sudden coronary thrombosis or spasm. Whilst *in vivo* studies of hearts *in situ* may be more clinically relevant to the human situation, isolated rat hearts have frequently been used to investigate ischaemia/reperfusion arrhythmias (Lubbe *et al*, 1978; Woodward and Zakaria, 1983; Hearse and Tosaki, 1987), since this model is free from extracardiac nervous reflexes and the influences of the general circulatory system. Although rat hearts are abnormal in that they do not exhibit an isoelectric ST segment (owing to the absence of a prolonged plateau phase of the action potential) and in their ability to spontaneously defibrillate (possibly related to their small size and hence relatively few electrical wavefronts) (Botting *et al*, 1986), they have continually proved to be a reliable and useful model for the study of arrhythmias.

The experiments detailed in this chapter were aimed at establishing control values for haemodynamic (described in subsequent chapters) and arrhythmia parameters during ischaemia and reperfusion in the

isolated rat heart. The effects of ischaemia and reperfusion on the efflux of potassium were monitored using $^{86}Rb^{+}$ as a radioactive marker for potassium. Since reperfusion is inevitably accompanied by the washout of substances accumulated in the ischaemic zone, a study was made of the time course of washout by measuring the appearance of lactate in coronary effluent. Myocyte damage was evaluated by measuring the release of the intracellular enzyme lactate dehydrogenase (LDH). It has already been mentioned that the xanthine/xanthine oxidase system is often used to produce oxygen free radicals (McCord and Fridovich, 1968), and the effects of this system on $^{86}Rb^{+}$ efflux were also investigated.

The rationale for measuring changes in the rate and magnitude of $^{86}Rb^{+}$ (potassium) efflux may be apparent when the potentially arrhythmogenic effects of these changes are considered. As briefly mentioned in chapter 1, several characteristics of non-nodal tissue action potentials, particularly the resting membrane potential, depend upon the relative potassium concentrations of the intracellular and extracellular compartments. Changes in the extracellular potassium concentration alter the potassium conductance of the sarcolemma (Weidmann, 1956), which in turn affects the plateau duration and the slopes of rapid repolarisation (phase 3) and spontaneous depolarisation (phase 4). Any combination of these changes may be arrhythmogenic or antiarrhythmic (Gettes, 1981).

3.2 RESULTS: THE ISOLATED PERFUSED RAT HEART. EFFECTS OF REPERFUSION FOLLOWING 10 MINUTES REGIONAL ISCHAEMIA

During the course of this study, the order of experiments was randomised to reduce the effects of daily variations in experimental conditions. This in turn necessitated the pooling of data for each experimental group, which for control hearts meant pooling data obtained over a period of approximately 2 years. However, statistical comparisons between control groups revealed insignificant differences between mean values of the parameters measured. For reasons of practicality, treatment groups were therefore compared with a single, large control group (n=43).

3.2.1 Reperfusion-induced Arrhythmias in Control Hearts

On reperfusion of control hearts, the incidences of VT and VF were 86% and 72% respectively. The mean onset of the first episode occurred after 6±1s for VT and 13±2s for VF, with a total duration of 16±3s and 75±13s respectively. The average total number of PVC's counted during the first 3 min of reperfusion was 199±30.

3.2.2 Reperfusion-induced $^{86}Rb^+$ Efflux in Control Hearts

During normal potassium (5.9mM K^+) and low potassium (3.2mM K^+) perfusion, the erc of $^{86}Rb^+$ remained at a level of approximately 0.040 min^{-1} (figure 3.2.2). During CAL, as might be expected following a reduction in the amount of tissue receiving flow, erc showed a gradual decline to a level around 0.030 min^{-1}. Immediately

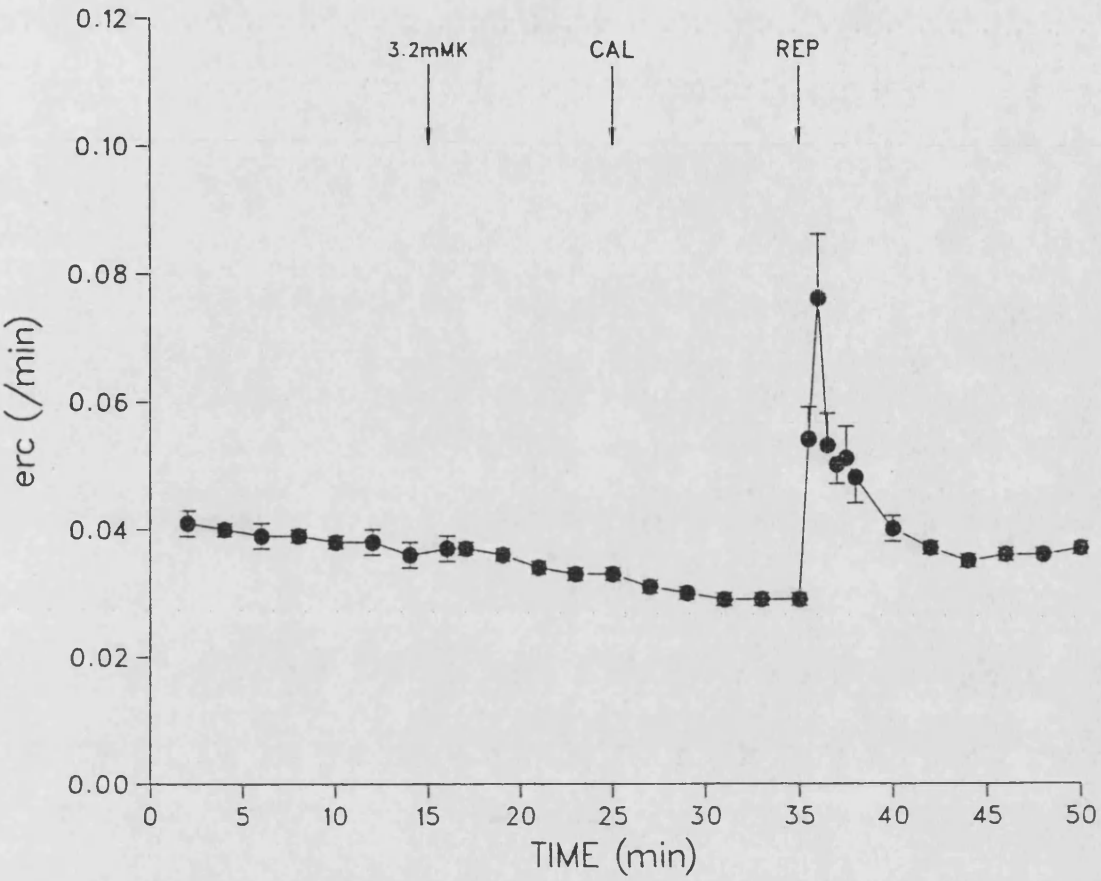


Figure 3.2.2

Reperfusion-induced rubidium-86 efflux in control hearts (erc/min).

Hearts were perfused with buffer containing 5.9 mM potassium for 15 min, and then with low (3.2 mM) potassium-containing buffer for the remainder of the experiment. 10 min after the start of low potassium perfusion, the coronary artery of each heart was ligated (CAL) for a further 10 min, followed by a 15 min period of reperfusion (REP).

Each point is the mean of 21 experiments. Vertical bars represent the standard error of each mean value.

after reperfusion, $^{86}Rb^{+}$ efflux rose sharply, reaching a mean peak value of 0.076±0.010 min^{-1}. For the first 3 min of reperfusion, the mean area under the efflux curve (which was used as an index of total $^{86}Rb^{+}$ efflux for this interval) was 0.133±0.023 arbitrary units (au). Figure 3.2.3 shows that a good correlation existed (r^2=0.86, n=21) between peak erc and efflux area for each heart. The time at which peak erc was attained for each heart did not vary. For control and all treated hearts, peak erc occurred during the second 30s period of reperfusion.

3.2.3 Reperfusion-induced Lactate Washout and LDH Release

Following reperfusion, there was a substantial release of $^{86}Rb^{+}$ (section 3.2.2). This may have been caused by myocardial cell membrane damage/dysfunction, or simply as a result of the washout of accumulated $^{86}Rb^{+}$ from the ischaemic area, where coronary flow during ischaemia was negligable. A seperate study was therefore undertaken to measure lactate and LDH in coronary effluent. Lactate is only produced during ischaemia and accumulates in the ischaemic zone (Neely and Feuvray, 1981) from where it is washed out during reperfusion. LDH however, is an intracellular enzyme that is only released following cell membrane damage. A comparison of the time course of lactate and LDH release with that of $^{86}Rb^{+}$ efflux would therefore provide evidence of a washout or reperfusion-induced release of the radioactive ion. Figure 3.2.4 illustrates the outcome of this study. As previously seen (section 3.2.2), $^{86}Rb^{+}$ efflux rose following reperfusion, reaching a mean peak value in this experiment of 0.075±0.005 min^{-1} after 1 min. Mean erc

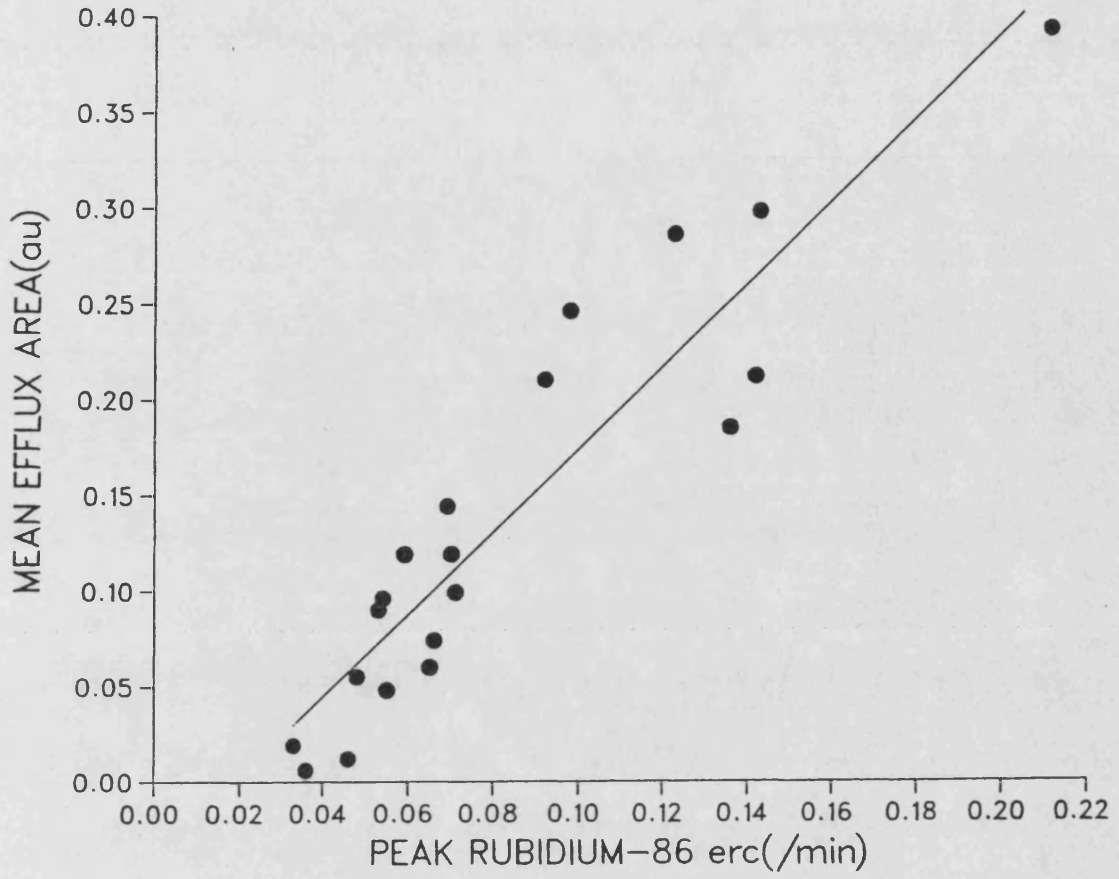


Figure 3.2.3

The relationship between the 2 parameters used to assess rubidium-86 efflux in control hearts.

Peak efflux rate coefficient (erc) values are plotted against area under the efflux curve during the first 3 min of reperfusion for individual hearts.

Least squares linear regression was performed on all data points resulting in a straight line plot with a regression coefficient (r^2) of 0.86.

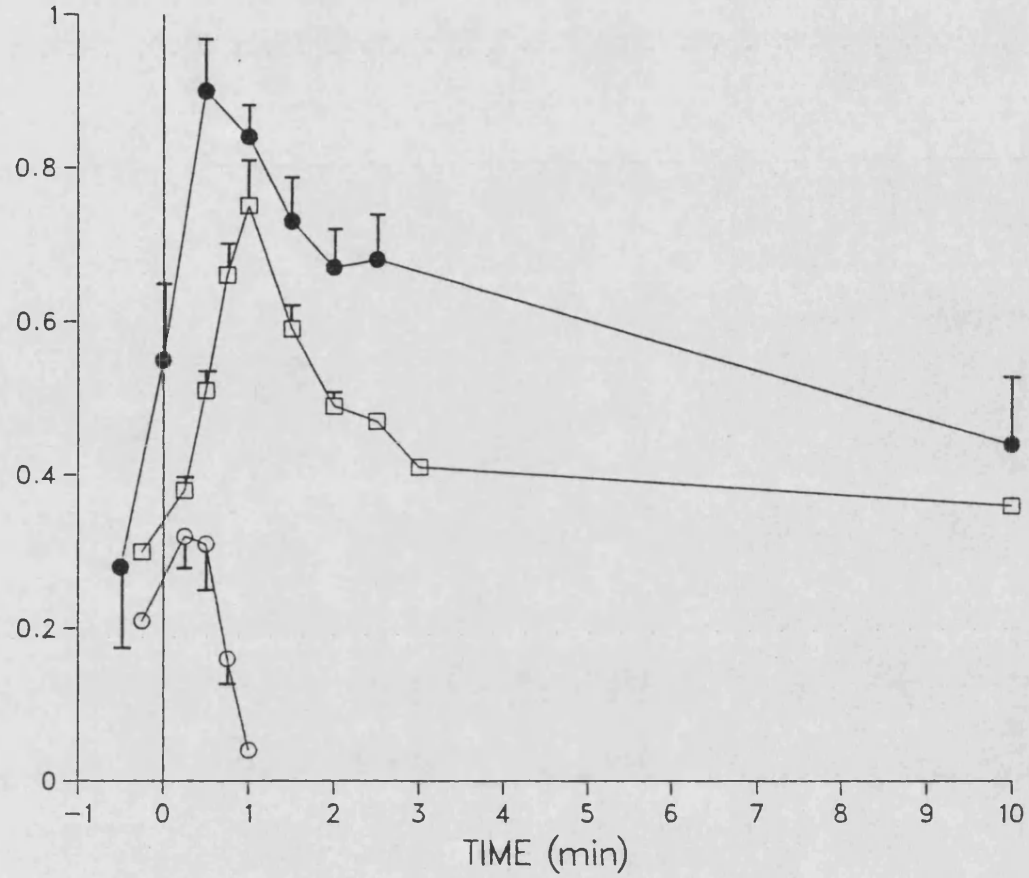


Figure 3.2.4

Lactate and lactate dehydrogenase concentrations and rubidium-86 erc in coronary effluent from control hearts.

Reperfusion was initiated at time 0 after a 10 min period of regional ischaemia.

Mean and standard errors are shown for lactate ○ (μmoles/ml/g wet weight, n = 6), lactate dehydrogenase ● (U/ml, n = 3) and rubidium-86 erc □ (min^{-1} x 10, n = 6).

approached a pre-reperfusion level towards the end of the 10 min reperfusion period. LDH release followed a similar time course profile. From a pre-reperfusion value of 0.28±0.11 U/ml, the concentration of LDH rose to a post-reperfusion peak of 0.90±0.07 U/ml 30s after reperfusion was initiated. At the end of the 10 min period, enzyme concentration had declined to 0.44±0.10 U/ml. Contrary to both $^{86}Rb^{+}$ and LDH profiles, the washout of lactate exhibited a more rapid time course. The concentration of lactate just before reperfusion was 0.21±0.01 μmoles/ml/g wet weight. Following reperfusion, this level had increased to a mean peak value of 0.32±0.04 μmoles/ml/g wet weight after only 15s. Lactate concentration had fallen below the ischaemic level 45s after reperfusion and was only 0.05±0.01 μmoles/ml/g wet weight after 1 min. These results strongly suggest that the efflux of $^{86}Rb^{+}$ following coronary artery reperfusion is not solely a passive washout response, but that there is a contribution to this effect from reperfusion-induced tissue "damage".

3.2.4 Free Radical Generation and $^{86}Rb^{+}$ Efflux

Since free radicals may be involved in reperfusion arrhythmogenesis (Woodward and Zakaria, 1985) and potassium loss has also been implicated (Lubbe *et al*, 1978), it was important to determine if free radicals could cause potassium ($^{86}Rb^{+}$) loss in a normal heart. In these experiments, superoxide radicals were generated using a xanthine/xanthine oxidase system (McCord and Fridovich, 1968). Hearts were perfused with xanthine (0.1mM) and a bolus injection of 0.01ml (0.65 U) xanthine oxidase administered. No significant

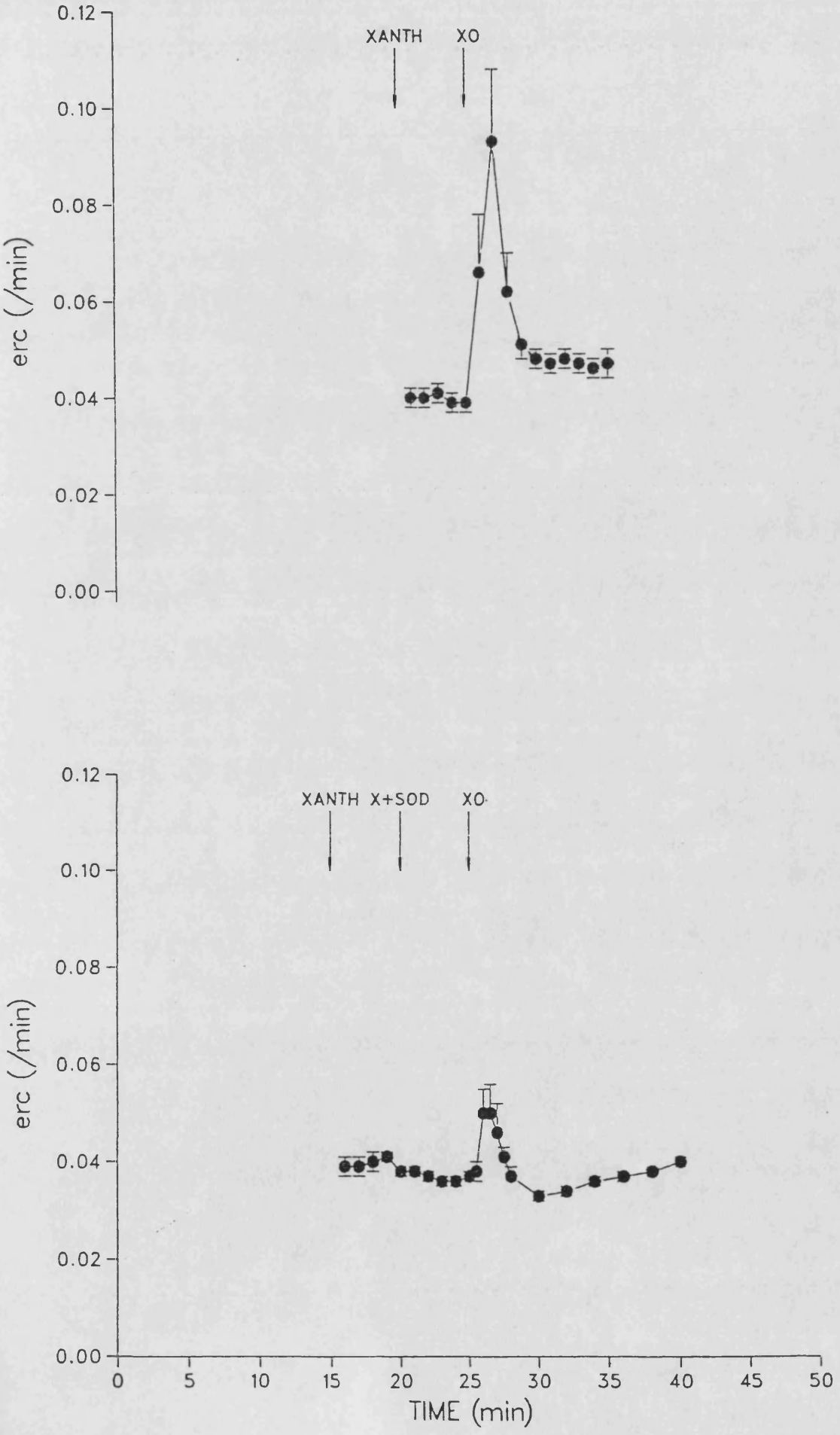
effect on mean $^{86}Rb^+$ erc was observed during xanthine perfusion (figure 3.2.5a). Administration of xanthine oxidase caused a mean increase in erc to a peak value of 0.093 ± 0.015 min^{-1}, rendering a mean efflux area (for the first 3 min following xanthine oxidase addition) of 0.073 ± 0.035 au (n=10). The magnitude of this response was reduced when superoxide dismutase (SOD), a superoxide radical scavenger, was perfused concomitantly with xanthine (figure 3.2.5b). Administration of xanthine oxidase under these conditions elevated mean $^{86}Rb^+$ erc to a peak value of only 0.050 ± 0.005 min^{-1}. Similarly, the corresponding efflux area was reduced to 0.045 ± 0.017 au (n=6). No significant effect on erc was brought about by perfusion with xanthine plus SOD. The above results provide evidence that the efflux of $^{86}Rb^+$, a radioactive marker for potassium, is stimulated by the xanthine/xanthine oxidase system probably as a result of superoxide radical generation. Since hearts were not ligated in these experiments, the involvement of $^{86}Rb^+$ accumulation and washout in this response was avoided. As reperfusion of ligated hearts also evoked a rise in $^{86}Rb^+$ efflux (section 3.2.1), there exists the possibility that reperfusion itself leads to the generation of free radicals. However, reperfusion of ligated hearts was also accompanied by the development of ventricular arrhythmias, a response that was conspicuously absent in the above experiments. One explanation for this is that xanthine oxidase-induced free radical generation led to a global elevation of $^{86}Rb^+$ efflux rather than an heterogeneous distribution of ions. Consequently, 6 hearts were perfused with 0.1mM xanthine and ligated prior to the administration of xanthine oxidase. Ligation was maintained throughout each experiment. Even

Figure 3.2.5:

The effects of free radical generation on $^{86}Rb^{+}$ erc in isolated rat hearts.

Radicals were generated by the administration of 0.01ml (0.65 U) xanthine oxidase (XO) in (a) hearts perfused with 0.1mM xanthine (XANTH, n=5) or (b) hearts perfused with 0.1mM xanthine plus SOD (X+SOD, n=6).

Mean and standard error values are shown.


XANTH
XO
0.12
0.10
0.08
0.06
0.04
0.02
0.00
erc (/min)
XANTH
X+SOD
XO
0.12
0.10
0.08
0.06
0.04
0.02
0.00
erc (/min)
0
5
10
15
20
25
30
35
40
45
50
TIME (min)

under these conditions however, xanthine oxidase did not evoke arrhythmias in any of the hearts. Assuming that heterogeneous radical generation leads to an heterogeneous distribution and localised elevation of potassium ions, there would appear from these results to be no relationship between ionic heterogeneity and arrhythmogenesis, although careful interpretation of these data is required. For example, free radicals, generated in these experiments after CAL, would be located in a much larger area of heart tissue (compared with reperfusion-generated radicals, which would be localised in the ischaemic zone) that is receiving normal and redirected coronary flow and that is likely to possess higher activities of endogenous free radical scavengers. In addition, the generation of free radicals in non-left ventricular and non-septal tissue may not be detrimental to normal cardiac electrophysiological function. A relationship between potassium distribution cannot therefore be resolved from these data, and further investigations are clearly required to resolve this anomoly.

3.3 DISCUSSION

The results presented confirm the findings of others (Lubbe *et al*, 1978; Woodward and Zakaria, 1983; Manning and Hearse, 1984) that reperfusion following transient coronary artery occlusion in isolated rat hearts leads to the genesis of ventricular tachyarrhythmias. Such observations may be extremely relevant to the clinical situation, since arrhythmias also arise during angioplastic or thrombolytic procedures (Goldberg *et al*, 1983) and have been proposed by some (Corr and Witkowski, 1983) to be the underlying cause of sudden cardiac death. Re-entry circuits are considered by many (Janse, 1982; Corr and Witkowski, 1983; Manning and Hearse, 1984) to be the mechanism by which ventricular fibrillation arises and are thought to result from the heterogeneic injury or recovery of myocardial tissue (Manning and Hearse, 1984). Arrhythmogenesis is undoubtedly a consequence of disturbances of ionic homeostasis (Bernier *et al*, 1986), and the present work has demonstrated that reperfusion is accompanied by a substantial efflux of $^{86}Rb^{+}$ (a radioactive tracer ion for potassium). This does not appear to be the result of washout of ions accumulated in the ischaemic region, as demonstrated by a comparison with the washout time profile of lactate. However, ligation of the left anterior descending coronary artery resulted in only a slight reduction of $^{86}Rb^{+}$ erc values (figure 3.2.2). It could be argued that the reduction in flow during CAL to an ischaemic area comprising 30-35% of the total ventricular wet weight might be expected to reduce $^{86}Rb^{+}$ erc values during this period by a similar magnitude. The fact that it did not requires explanation. In the constant flow

isolated heart model, ligation of the coronary artery will redirect flow from the vascular bed served by that artery to the non-ischaemic vasculature. This was routinely observed as an increase in perfusion pressure (immediately upon ligation) to values between 100 and 150 mmHg. Similar flow (and consequently pressure) increases were demonstrated by Zakaria (unpublished results) not to cause vascular damage, as indicated by the absence of protein loss following ligation. However, the effects of such changes on potassium efflux have not been determined, and it is possible that elevated vascular pressure increases efflux either by mechanically squeezing adjacent tissue or by perfusing capillaries that are underperfused during normal flow conditions. Whilst pressure-induced increases in potassium efflux remain to be shown, it is conceivable that such a phenomenon could compensate for the reduced efflux expected during coronary ligation.

The mechanism responsible for the elevation of $^{86}Rb^{+}$ efflux has not been determined. The observed response may be caused by a reduced influx of $^{86}Rb^{+}$ resulting from inhibition of the sarcolemmal sodium-potassium pump. Further experiments aimed at monitoring sodium fluxes during reperfusion simultaneous with measurements of $^{86}Rb^{+}$ efflux would give some indication of sodium-potassium pump activity and may elucidate the role of this system in the observed effects on $^{86}Rb^{+}$ movements. Other mechanisms have been proposed to account for the elevation of extracellular potassium concentrations in ischaemic myocardium (Gaspardone *et al*, 1986). Acidosis develops rapidly during ischaemia (Hirche *et al*, 1980) and whilst imposed acidosis (increased pCO_2 of perfusate) has been shown to alter

myocardial potassium fluxes (Poole-Wilson and Langer, 1975), these changes comprised a net gain of potassium by the myocardium rather than a loss. Potassium loss may also be mediated by an increase in membrane permeability, and reductions in cellular ATP levels during ischaemia could lead to the opening of potassium channels which rely on ATP to maintain their "closed" state (Noma, 1983).

Tranum-Jensen and colleagues (1981) measured an increased cellular osmolarity in ischaemic cells that has since been implicated by Gaspardone *et al* (1986) as a possible mechanism by which potassium is lost. However, the latter group demonstrated an increased uptake of potassium in rabbit interventricular septa perfused with hyperosmolar solutions which reverted to a net loss of potassium when isosmolar perfusion was resumed. The authors suggested that the enhanced influx was the result of potassium exchanging with sodium that had left the cell to reduce osmolarity. They proposed that reperfusion of ischaemic tissue normalises osmolarity, reduces potassium influx and produces a net loss of intracellular potassium. More importantly, there was no evidence that potassium efflux increased when isosmolar perfusate was reintroduced. It therefore seems unlikely that changes in the osmolarity of ischaemic cells following reperfusion were responsible for the large rises in $^{86}Rb^{+}$ efflux observed in the present study.

Patch-clamp experiments have shown the existence of ATP-regulated potassium channels in cardiac sarcolemma that are activated when intracellular ATP levels fall (Trube and Hescheler, 1984). Whilst potassium channel activation during coronary reperfusion has not,

to the author's knowledge, been investigated, such activation may be responsible for the reperfusion-induced elevation of $^{86}Rb^{+}$ efflux described earlier. Further studies using potassium channel blocking drugs under conditions of ischaemia/reperfusion would elucidate whether $^{86}Rb^{+}$ efflux elevation is a channel-mediated phenomenon. Several studies, more recently those of Bacaner and colleagues (1986), have used potassium channel blockade in dog hearts *in situ* to successfully increase the ventricular fibrillation threshold (i.e. the lowest current required to induce sustained fibrillation). The converse situation (a decrease in ventricular fibrillation threshold) was achieved by Logic (1973) by the intracoronary infusion of hyperkalaemic solutions in the same model. Experiments such as these illustrate the importance of cellular potassium ion gradients to the vulnerability of hearts to ventricular fibrillation.

Bretylium
Bethanidine

In experiments reported earlier, superoxide radical ($O_2\cdot^-$) generation with xanthine/xanthine oxidase reproduced the elevation of $^{86}Rb^{+}$ efflux seen when hearts were reperfused. This effect was reduced in the presence of SOD and is abolished (Zakaria, 1985) when inactivated (boiled) xanthine oxidase is used. Although these results demonstrate the involvement of $O_2\cdot^-$ in elevating $^{86}Rb^{+}$ efflux in non-ligated hearts, it would be premature to extrapolate this mechanism to the reperfused heart. Whilst $O_2\cdot^-$ has been shown to inhibit sodium-potassium ATPase (Na^{+}-K^{+} ATPase) in brain capillaries (Lo and Betz, 1986) and rat lung (Das and Neogi, 1984), a similar effect was not investigated in the current study. Furthermore, inhibition of the Na^{+}-K^{+} pump has only been observed

after 30 min of regional ischaemia (Kleber, 1983), although there is no reason to believe that reperfusion-induced events follow similar mechanisms to those occurring during ischaemia. Clearly then, a study of Na^+-K^+ ATPase activity during reperfusion is required.

The objectives stated at the beginning of this chapter have generally been achieved. Responses of isolated hearts in terms of $^{86}Rb^+$ efflux following reperfusion bare a close similarity to those obtained by the enzymic generation of $O_2\cdot^-$, with the exception that the latter procedure did not evoke arrhythmias. Reasons for this anomoly can only at present be speculative. A global generation of free radicals lacks the heterogeneity expected of regional ischaemia and reperfusion, resulting in a situation that does not tend to favour re-entry. In addition, a period of oxygen deprivation may be a pre-requisite for free radical-mediated damage during oxidative stress. This possibility is supported by observations of reduced SOD and glutathione peroxidase activities during hypoxia (Guarnieri *et al*, 1980) and ischaemia (Julicher *et al*, 1984). It is therefore possible that by simply generating free radicals in the absence of any ischaemic insult and under non-heterogeneic conditions, myocyte antioxidant defences may not be sufficiently overwhelmed to result in perturbations of ionic homeostasis of arrhythmogenic proportions.

Finally, despite the circumstantial evidence supporting a relationship between reperfusion-induced arrhythmias, $^{86}Rb^+$ efflux and oxygen free radical generation presented here, the need for

data to reinforce this hypothesis is clearly apparent. An attempt to identify whether such a relationship exists is described in the following chapter.

CHAPTER 4

4.1 INTRODUCTION: PUTATIVE ENDOGENOUS FREE RADICAL SYSTEMS AND PHARMACOLOGICAL INTERVENTION

Before free radicals can be implicated as arrhythmogenic factors during coronary reperfusion, the criterion must be fulfilled that inhibition or removal of the source of free radicals reduces the severity of rhythm disturbances. Since it has been demonstrated (chapter 3) that free radical generation increases the efflux of $^{86}Rb^{+}$, a response also obtained following reperfusion, there is some indication that potassium fluxes are involved in arrhythmogenesis and may be modified by free radicals. With these considerations in mind, it was considered expedient to study the effects of pharmacological intervention on several putative endogenous free radical generating systems with regard to the severity of arrhythmias and the degree of $^{86}Rb^{+}$ efflux following reperfusion.

Reduced glutathione (GSH) has been shown to protect against reperfusion-induced arrhythmias (Woodward and Zakaria, 1985; Bernier *et al*, 1986) at concentrations within the normal physiological range for heart tissue (Kosower and Kosower, 1978). A reduction of the cellular GSH content is detrimental to many physiological processes, including intracellular calcium sequestration (Jones *et al*, 1973; Bellomo *et al*, 1983; Thor *et al*, 1985), calcium ATPase activity (Nicotera *et al*, 1985), drug metabolism (Orrenius *et al*, 1984), polyamine biosynthesis (Guarnieri *et al*, 1982) and antioxidant inhibition of lipid peroxidation (Barsacchi *et al*, 1984). The importance of GSH in

GSH released – not GSSG
GSH Px activity ↓↓

protection against myocardial reperfusion damage is implicated by many studies which show a reduction of cardiac GSH levels during oxidant stress (Guarnieri *et al*, 1979; Doroshow *et al*, 1980), cardiac reperfusion (Ferrari *et al*, 1985; Kajiyama *et al*, 1987) and in endothelial cell cultures exposed to H_2O_2 (Harlan *et al*, 1984; Tsan *et al*, 1985). One aim of this study was therefore to examine the responses of isolated hearts to GSH perfusion in terms of rhythm disturbance and $^{86}Rb^+$ efflux.

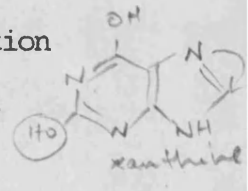
Endogenous systems capable of generating oxygen free radicals were reviewed in chapter 1, and the role of transition metals in these processes was emphasized. The participation of iron in free radical generating systems is often studied by removing free iron with chelating agents (Halliwell and Gutteridge, 1985). The most commonly used agent for chelating metals is ethylenediamine-tetraacetic acid (EDTA). However, for the removal of iron from free radical reactions, EDTA is inadequate because the resultant Fe^{3+}-EDTA complex can still be reduced by $O_2\cdot^-$ (Butler and Halliwell, 1982). EDTA is also a strong chelator of calcium and may therefore exert other non-specific effects. More commonly used is desferrioxamine, an iron chelator extracted from the bacterium *Streptomyces pilosus*. Desferrioxamine is a linear molecule containing three N-O and three C=O groups. One molecule binds one Fe^{3+} ion by bending round the six oxygen ligands to form an Fe^{3+}-desferrioxamine complex. The Fe^{3+} ions are bound tightly so that they cannot be reduced by $O_2\cdot^-$. Desferrioxamine is relatively specific for Fe^{3+} ions (although it will bind Al^{3+} ions also) and is commonly used in the treatment of beta-thalassaemia and other

diseases that lead to iron overload (Halliwell and Gutteridge, 1985). Desferrioxamine was therefore employed in the present study to investigate the effects of iron chelation on reperfusion arrhythmias and $^{86}Rb^{+}$ efflux in isolated rat hearts.

The elevated myocardial concentration of noradrenaline during ischaemia and reperfusion (section 1.5.3) has been implicated as an arrhythmogenic factor (Schomig *et al*, 1984). Woodward and Manning (1987) have suggested that "the lack of a consistent picture regarding the antiarrhythmic properties of alpha- and beta-receptor antagonists points to alternative mechanisms by which noradrenaline may be arrhythmogenic". One possibility is the generation of free radicals resulting from catecholamine autoxidation. Indeed, Zakaria (1985) was able to abolish the reperfusion-induced reduction of ferricytochrome c (an assay for $O_2 \cdot^-$) by depleting rats of catecholamines using 6-hydroxydopamine (6-OHDA). This method was therefore employed in the present study to examine the effects of catecholamine depletion on the incidence of arrhythmias and the efflux of $^{86}Rb^{+}$ following reperfusion of isolated hearts.

The involvement of xanthine oxidase in free radical-mediated reperfusion damage has been extensively studied in the intestine (Parks and Granger, 1983; Roy and McCord, 1983; Granger *et al*, 1986). Such studies have provided a basis for investigations of the enzyme's involvement in myocardial damage, and this is considered in detail in chapters 1 and 6. However, an aim of the work presented in this chapter was to assess the contribution of xanthine oxidase-derived free radicals to reperfusion-induced

arrhythmias and $^{86}Rb^{+}$ efflux. For this purpose, the xanthine oxidase inhibitors allopurinol and oxypurinol were employed. Allopurinol is an antihyperuricaemic agent, but has also been shown experimentally to reduce myocardial infarct size (Akizuki *et al*, 1985) and attenuate free radical-induced increases of vascular permeability (Korthuis *et al*, 1985). Whilst allopurinol's mode of action in these pathological conditions is uncertain, the drug has been demonstrated to dilate coronary collateral vessels (Arnold *et al*, 1980) and preserve tissue levels of high energy adenine nucleotides (De Wall *et al*, 1971). Allopurinol's inhibitory action on xanthine oxidase has been elucidated. Spector and co-workers (1986) described allopurinol as a "suicide" substrate for the enzyme. It becomes oxidised to oxypurinol which binds tightly to xanthine oxidase and competitively inhibits the enzyme (overall inhibition constant = 85nM-100nM). In 1984, Manning and colleagues demonstrated a reduction of the incidence of reperfusion-induced ventricular fibrillation in *in situ* hearts of rats treated with 20mg/kg allopurinol. In isolated rat hearts, Zakaria (1985) was unable to show a significant reduction of arrhythmias following reperfusion when allopurinol (0.1mM) was perfused through hearts 5 min before CAL. It is possible that in the latter experiments, insufficient time was allowed for the conversion of allopurinol to oxypurinol (the active inhibitor of xanthine oxidase), although the time required for this process has not been reported. On the basis of these findings, it seemed important to determine whether the antiarrhythmic effects of allopurinol pre-treatment could be reproduced by perfusion of oxypurinol through hearts prior to CAL/reperfusion.



Since reperfusion arrhythmias can be reduced in isolated hearts by SOD (Woodward and Zakaria, 1985), implicating a role for $O_2\cdot^-$ in this response, it is proposed that inhibition of endogenous SOD should exacerbate the incidence of arrhythmias on reperfusion. A method for the inhibition of SOD, using the powerful copper chelating agent diethyldithiocarbamate (DDC), is available (Heikkila *et al*, 1976) and has been shown to reduce cardiac SOD activity in rats by 93% at a dose of 1.2g/kg ip (Rossoni Caldarera *et al*, 1982). The activity of DDC is ascribed to its ability to chelate the copper of Cu-Zn SOD, since Heikkila and colleagues were able to restore SOD activity by the addition of copper sulphate solution. The Cu^{2+} ions were thought to compete with the enzymic copper for DDC, thus removing DDC and restoring enzyme activity. DDC may also be active on Mn SOD, since Rossoni Caldarera *et al* (1982) demonstrated a reduction of respiratory function in mitochondria extracted from the hearts of rats pre-treared with DDC. The authors attributed the reduced mitochondrial function to the accumulation and damaging effects of $O_2\cdot^-$, which had not been neutralised by the inhibited SOD. However, their assumption that Mn SOD was the inhibited enzyme may have been premature. Steinman (1982) has demonstrated that Cu-Zn SOD is also present between the inner and outer mitochondrial membranes, and it is possible that it was this form of the enzyme that was inhibited by Rossoni Caldarera's group. DDC was used in the present study to inhibit SOD activity and examine the effects of this inhibition on reperfusion arrhythmias and $^{86}Rb^+$ efflux. Enzyme inhibition was confirmed in a cell-free system by comparing the reduction of $O_2\cdot^-$ production (indicated by the reduction of ferricytochrome c) by SOD in the

presence and absence of DDC.

The final objective to be described in this chapter was to assess the importance of oxygen on the parameters studied. Hearse *et al* (1973) demonstrated that reoxygenation of hypoxically perfused hearts results in myocardial damage characterised by the release of intracellular enzymes. Zakaria (1985) extended this study by reperfusing regionally ischaemic isolated rat hearts with anoxic buffer and was able to show a reduced incidence of reperfusion arrhythmias compared with hearts reperfused with oxygenated buffer. These results contradict the findings of Petropoulos and Meijne (1964) who reported that reperfusion of ischaemic myocardium with venous blood or anoxic buffer did not influence the incidence of ventricular fibrillation during reperfusion. Experiments were therefore performed in an attempt to resolve the equivocalities of these studies, the results of which are described below.

4.2 RESULTS: GLUTATHIONE

Three concentrations of the free radical scavenger reduced glutathione (GSH), 0.01mM, 0.10mM and 1mM, were perfused through isolated hearts. Effects on arrhythmias, $^{86}Rb^{+}$ efflux and cardiac haemodynamics were investigated and compared with those of the control group.

4.2.1 Arrhythmias

All concentrations of GSH reduced the incidences of VT and VF following reperfusion (figure 4.2.1). From a control incidence of 86%, VT was reduced to 60%, 58% and 78% by 0.01mM, 0.10mM and 1mM GSH respectively. Statistical significance was only attained with 0.10mM GSH. The control incidence of VT (72%) was reduced to 60% by 0.01mM GSH, 42% by 0.10mM GSH and 48% by 1mM GSH; the last two reductions were significant when compared with the control group.

The effects of GSH on the onset and duration of arrhythmias are shown in table 4.2.1. The only significant change was a delay in the onset of VT in the presence of 1mM GSH. Compared to the control value (6 ± 1s), VT onset was delayed until 15 ± 6s after reperfusion. The number of PVC's that occurred during the first 3 min of reperfusion was unaffected by all concentrations of GSH.

4.2.2 $^{86}Rb^{+}$ Efflux

Reperfusion-induced $^{86}Rb^{+}$ efflux in control and GSH-treated hearts

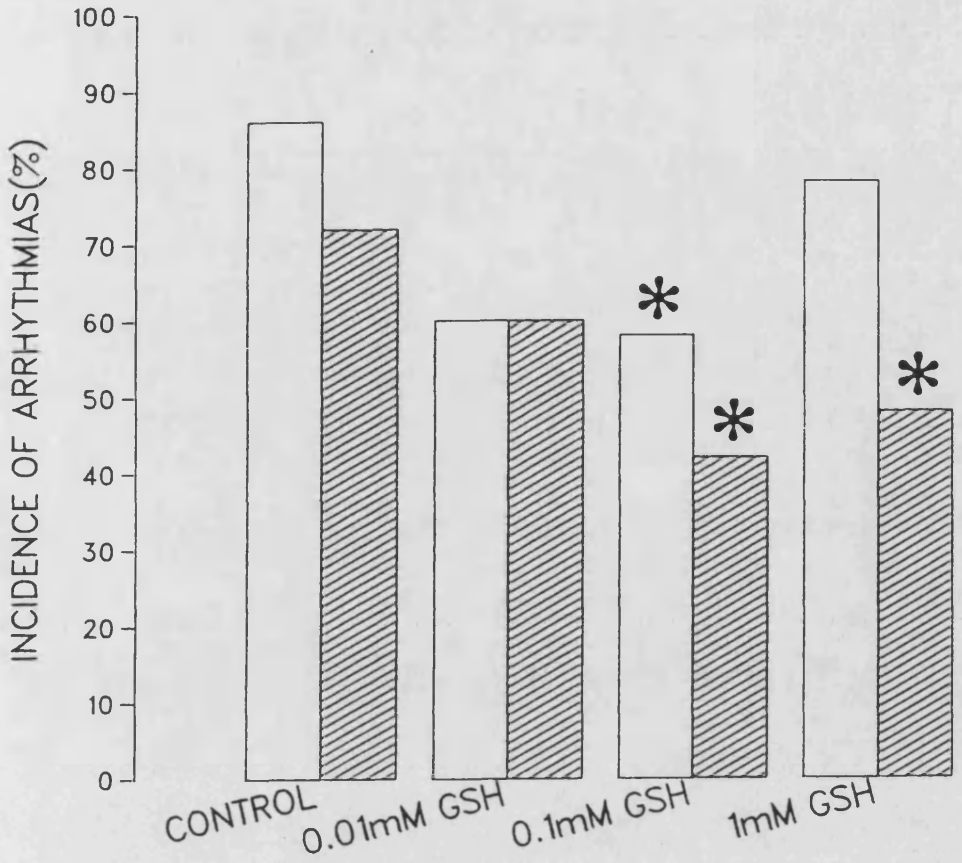


Figure 4.2.1

The percentage incidence of VT (open bars) and VF (shaded bars), following reperfusion, in control hearts (n = 43) and hearts perfused with glutathione (GSH).

0.01 mM GSH, n = 10; 0.10 mM GSH, n = 12; 1 mM GSH, n = 27.
* $p \leqslant 0.01$ compared with the control group.

		VT			VF			PVC's
	n	n	ONSET (sec)	DURATION (sec)	n	ONSET (sec)	DURATION (sec)	
CONTROL	43	37	6± 1	16± 3	31	13± 2	75±13	199±30
GSH 10^{-5}M	10	6	3± 1	33± 8	6	11± 2	25±10	166±80
10^{-4}M	12	7	11± 4	13± 4	5	25±11	49±32	111±38
10^{-3}M	27	21	15± 6*	16± 5	13	19± 4	75±15	121±25

Table 4.2.1:

The onset and duration of VT and VF and the number of PVC's during the first 3 min of reperfusion in control and glutathione-treated (GSH) hearts.

* $p<0.05$ compared with the control value.

is shown in figure 4.2.2. Mean peak efflux and mean efflux area in control hearts (0.076±0.010 min^{-1} and 0.133±0.023 au respectively) were significantly reduced only by 1mM GSH. In this group, mean peak $^{86}Rb^{+}$ efflux was 0.044±0.002 min^{-1} and mean efflux area fell to a value of 0.039±0.007 au.

4.2.3 GSH-related Haemodynamic Changes

Changes in heart rate (HR), developed tension (dT) and perfusion pressure (PP) following perfusion with 0.01mM GSH, 0.10mM GSH and 1mM GSH are presented in figure 4.2.3. The data shown represent the differences between pre- and post-drug values. HR was significantly reduced by 10±4 bpm and 12±3 bpm with 0.01mM GSH and 0.10mM GSH respectively. Significant reductions of dT were observed with 0.10mM GSH (0.61±0.36g) and 1mM GSH (0.91±0.28g). GSH also exhibited a concentration-dependent vasodilator action. 0.01mM, 0.10mM and 1mM concentrations caused PP to fall by 8±2mmHg, 20±3mmHg and 29±2mmHg respectively.

4.3 RESULTS: OTHER PHARMACOLOGICAL INTERVENTIONS

The relationship between arrhythmogenesis and potassium loss was again studied using interventions thought to affect free radical generation. The drugs used were desferrioxamine, 6-hydroxydopamine (6-OHDA), allopurinol, oxypurinol and diethyldithiocarbamate (DDC). Experiments were also carried out using hypoxic perfusate.

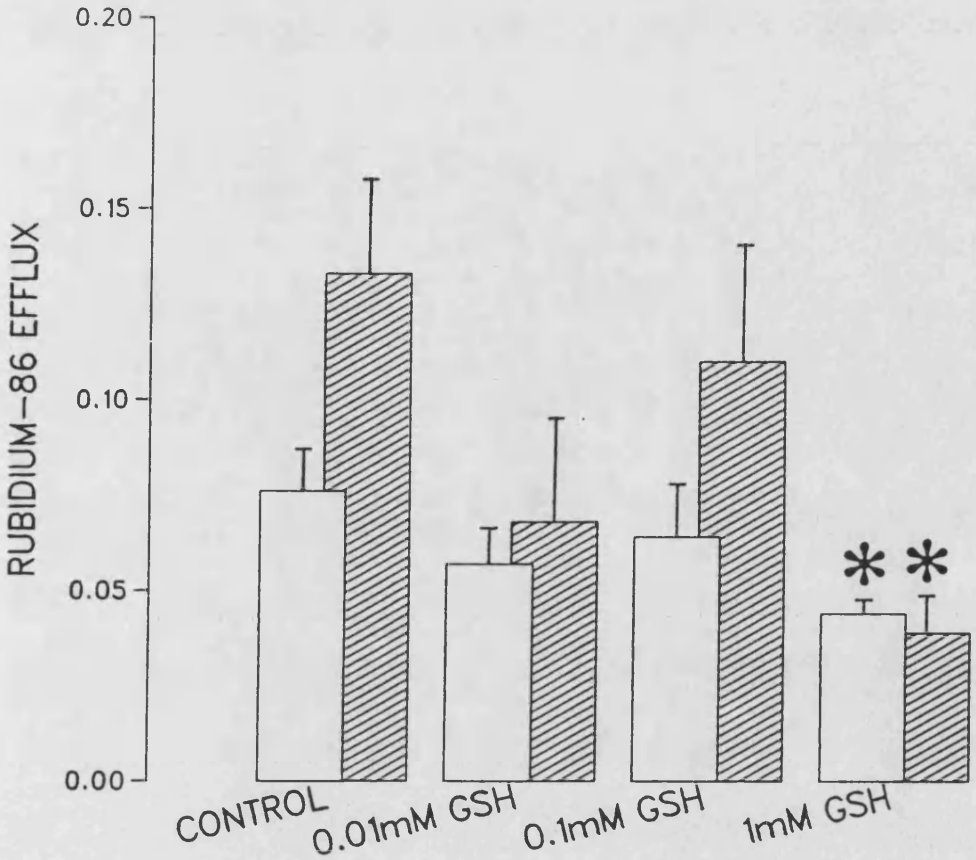


Figure 4.2.2

Reperfusion-induced rubidium-86 efflux in control (n = 21) and glutathione (GSH)-perfused hearts.

Vertical bars represent mean peak erc (min^{-1}; open bars) and mean efflux area (au; shaded bars), with corresponding standard errors (vertical lines), for the first 3 min of reperfusion.

0.01 mM GSH, n = 6; 0.10 mM GSH, n = 5; 1 mM GSH, n = 21.
*$p \leqslant 0.05$ compared with the control group.

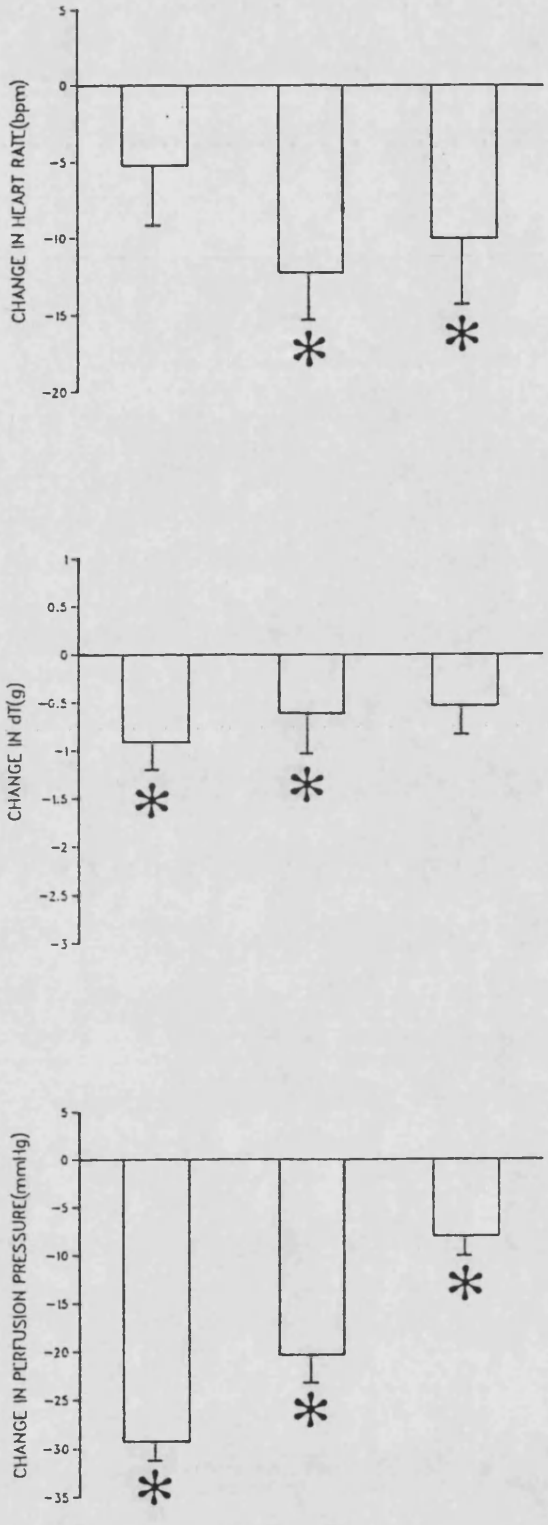


Figure 4.2.3:

Haemodynamic responses of isolated rat hearts to glutathione (GSH).
Results are expressed as the mean and standard error of the change following GSH perfusion.

*p<0.05 compared with the pre-treatment value.

Mean pre-treatment values: HR: 256±3 bpm (n=61)
dT: 8.3±0.1g (n=61)
PP: 89±4 mmHg (n=61)

4.3.1 Arrhythmias

(a) Desferrioxamine:

At concentrations of 0.1mM and 1mM, desferrioxamine did not significantly affect the incidences of VT or VF (figure 4.3.1). Similarly, the onset and duration of arrhythmias and the number of PVC's observed following reperfusion were not statistically different from control values (table 4.3.1).

(b) 6-OHDA:

To investigate the possible arrhythmogenic role of free radicals generated by the autoxidation of myocardial noradrenaline, rats were pre-treated with 6-OHDA (60mg/kg iv) 24 hours before hearts were excised. 6-OHDA destroys sympathetic nerve terminals causing the release, oxidation and subsequent depletion of catecholamines. Successful administration of 6-OHDA was apparent from the appearance of treated animals, namely pilo-erection, peripheral cyanosis and lethargy. However, depletion of catecholamines was confirmed pharmacologically in hearts prior to each experiment. A bolus administration of tyramine (0.1ml, 0.1mM), an indirect sympathomimetic drug that displaces noradrenaline from intracellular stores, was without effect in all 6-OHDA-treated hearts, indicating successful depletion. Non-treated control hearts, when administered the same dose of tyramine, exhibited transient increases in HR, dT and PP of 43$\pm$3 bpm, 0.7$\pm$0.1g and 5$\pm$2mmHg respectively (n=3).

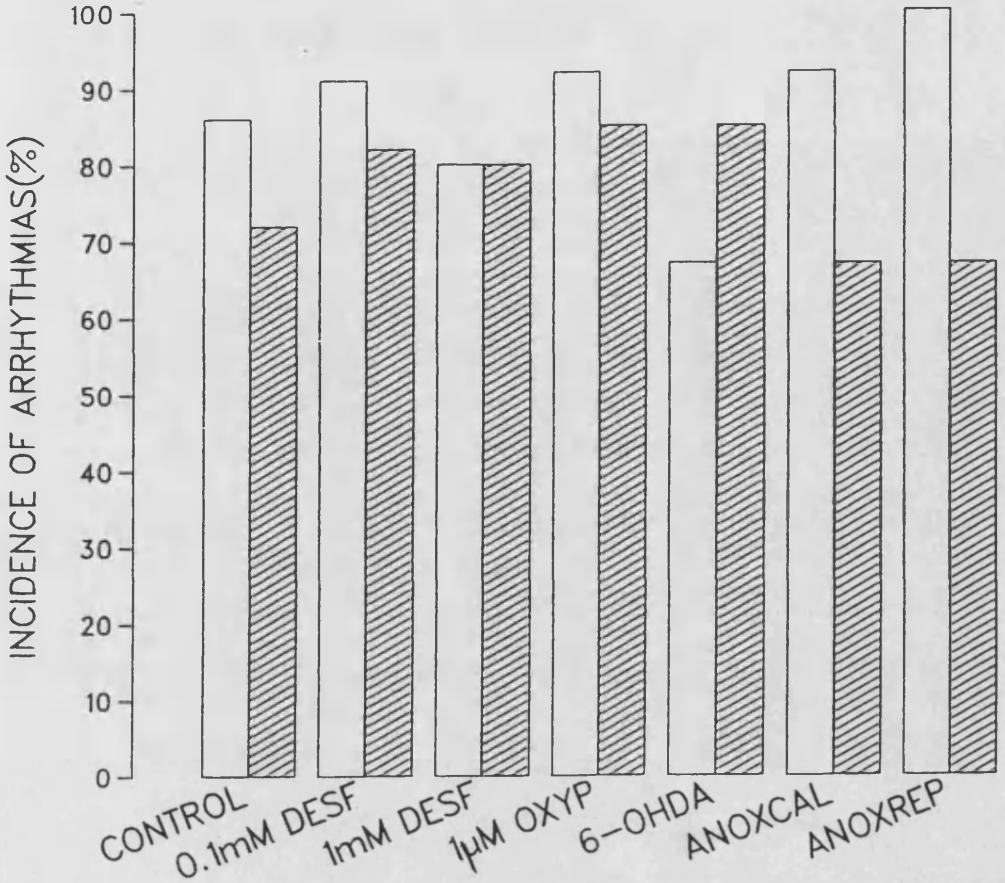


Figure 4.3.1:

The percentage incidence of VT (open bars) and VF (shaded bars) following reperfusion in isolated rat hearts.

CONTROL (n=43); DESF, desferrioxamine (0.1mM n=11, 1mM n=10); OXYP, oxypurinol (1μM n=13); 6-OHDA, 6-hydroxydopamine (60mg/kg n=12); ANOXCAL, hypoxically perfused from 2 min before CAL (n=12); ANOXREP, hypoxically perfused from 2 min before reperfusion (n=12).

* $p<0.05$ compared with the control group.

		VT			VF			PVC's
	n	n	ONSET (sec)	DURATION (sec)	n	ONSET (sec)	DURATION (sec)	
CONTROL	43	37	6± 1	16± 3	31	13± 2	75±13	199±30
DESF:								
10^{-4}M	11	10	5± 1	9± 1	9	12± 1	106±27	72±25
10^{-3}M	10	8	3± 1	12± 3	8	11± 2	96±28	105±14
OXYP:								
10^{-6}M	13	12	2± 1*	17± 5	11	13± 3	101±21	195±58
6-OHDA:								
60mg/kg	12	8	22±14*	14± 4	6	13± 3	44±28	138±44
ANOXCAL	12	11	13± 8*	23± 4*	8	27± 8*	153± 8*	321±54*
ANOXREP	12	12	2± 1	12± 2	8	22± 9	143±16	211±42*
SALINE	10	9	3± 1	6± 1	9	9± 1	50±23	97±26
ALLO	11	9	4± 1	9± 3	2	7± 2	173± 2*	123±44
SALINE	11	7	2± 1	13± 2	6	11± 2	8± 2	136±31
DDC	12	12	5± 3	11± 3	6	9± 2	19± 4*	161±34

Table 4.3.1:

The onset and duration of VT and VF and the number of PVC's during the first 3 min of reperfusion in control and drug- treated hearts.

DESF, desferrioxamine; OXYP, oxypurinol; 6-OHDA, 6-hydroxydopamine; ANOXCAL, hypoxically perfused from 2 min before CAL; ANOXREP, hypoxically perfused from 2 min before reperfusion; ALLO, allopurinol (20mg/kg); DDC, diethyldithiocarbamate (1.5g/kg).

* $p<0.05$ compared with the appropriate control value.

Although the incidences of VT and VF in hearts from treated animals were reduced (67% and 50% respectively), these changes were not statistically significant (figure 4.3.1). The onset of VT was significantly delayed however, from the control mean value of 6±1s to 22±14s. None of the other parameters was significantly affected (table 4.3.1).

(c) Hypoxic perfusion:

The effect of oxygen deprivation on arrhythmogenesis and $^{86}Rb^{+}$ efflux was tested by perfusing hearts with buffer rendered hypoxic by gassing with 95% N_2/5% CO_2. Hypoxic perfusion was initiated 2 min before CAL (ANOXCAL) and, in a separate series of experiments, 2 min before the onset of reperfusion (ANOXREP). Figure 4.3.1 illustrates the arrhythmic responses of hearts under these conditions.

The incidences of VT and VF were not significantly affected by either protocol. However, when hypoxia was induced before CAL, both the onset of VT (13±8s) and VF (27±8s) were significantly delayed when compared to the control values (6±1s and 13±2s respectively; table 4.3.1). The total duration of each type of arrhythmic episode was prolonged (23±4s for VT and 153±8s for VF). The number of PVC's was also increased to 321±54. No significant changes to onset times and durations of VT or VF were observed when hypoxic perfusion began 2 min prior to reperfusion, although the number of PVC's was again increased, this time to 211±42.

It was therefore apparent that hypoxia did not protect against

ventricular tachyarrhythmias, but rather delayed and prolonged arrhythmic episodes and increased the number of spontaneous PVC's.

(d) Allopurinol and oxypurinol:

Rats were pre-treated with allopurinol (20mg/kg ip 48hours, iv 15 min; a dose shown by Manning *et al* (1984) to reduce reperfusion arrhythmias in anaesthetised, open-chest rat heart preparations) dissolved in 1M NaOH and diluted with perfusion buffer (final pH = 8). Control rats were treated identically with an equivalent volume of 0.9% (w:v) saline (pH 8).

Figure 4.3.2 shows that the control incidences of VT and VF were both 90%. Hearts from allopurinol-treated animals exhibited a significant reduction of VF incidence to a value of 18%. The incidence of VT was not significantly altered. Despite this marked antifibrillatory effect, the duration of VF was more than triple that of the control group (173$\pm$2s compared with 50$\pm$23s; table 4.3.1).

Inhibition of xanthine oxidase by allopurinol is mediated by its oxidised metabolite oxypurinol (Spector *et al*, 1986). Consequently, the effect of oxypurinol on arrhythmogenesis was investigated by perfusing the drug through hearts. A concentration of 1μM was used, since Spector and colleagues (1986) demonstrated an overall inhibition constant, K_I, for oxypurinol of 85-100nM. In view of the above allopurinol data, the lack of protection against arrhythmias afforded by oxypurinol was surprising (figure 4.3.1). Neither the incidence of VT or VF was significantly reduced from control

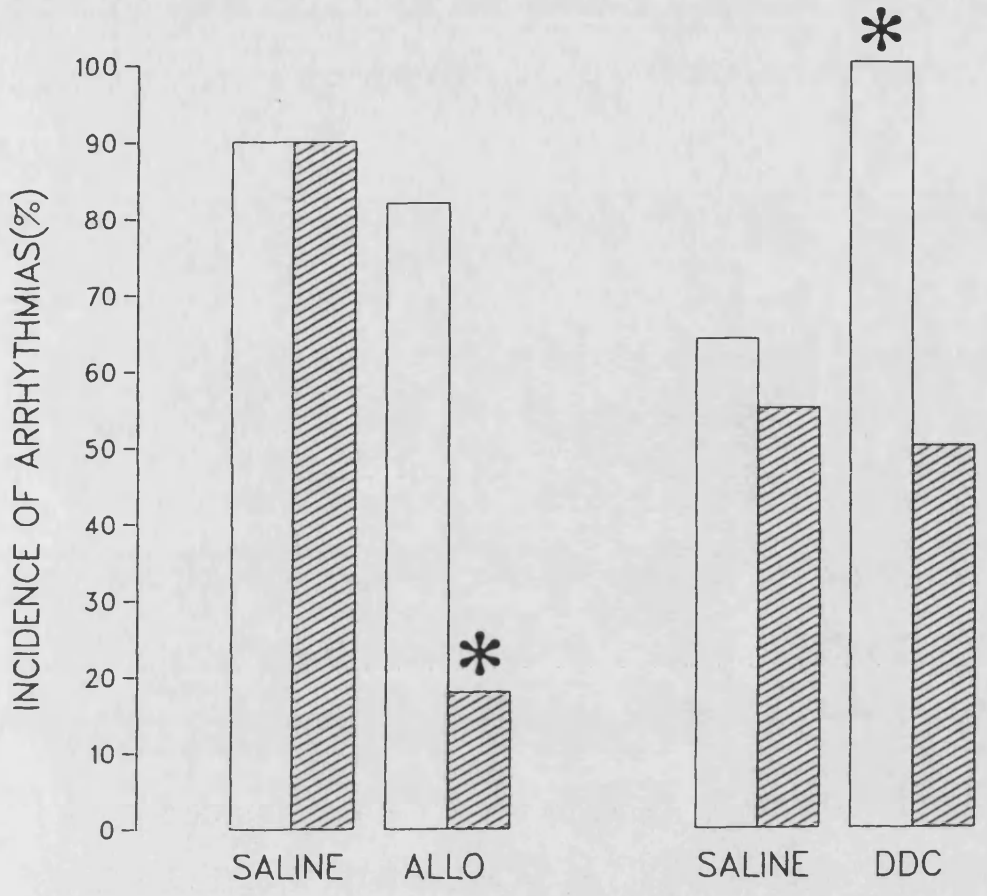


Figure 4.3.2:

The percentage incidence of VT (open bars) and VF (shaded bars) following reperfusion in hearts from rats pre-treated with allopurinol (ALLO, 20mg/kg), diethyldithiocarbamate (DDC, 1.5g/kg), or equivalent volumes of saline (0.9% w:v).

Hearts from rats pre-treated with DDC were perfused with buffer containing 5.9mM potassium to reduce the control incidence of arrhythmias (see section 4.3.1(e)).

* $p<0.05$ compared with the appropriate saline control group.

values. Furthermore, episodes of VT occurred earlier (2±1s) following oxypurinol perfusion than in the control group (6±1s).

The discrepancy between the antiarrhythmic action of allopurinol and a failure to protect against arrhythmias with oxypurinol may be the result of a direct antiarrhythmic effect of the former drug rather than an anti-free radical mechanism. Ideally, an investigation of the electro-physiological effects of allopurinol on the cardiac action potential would have demonstrated any such direct action. However, since this was not possible in our laboratory, an attempt to reveal any class I antiarrhythmic property was made by inducing VF in hearts using an "arrhythmogenic" perfusion fluid of low potassium, zero magnesium and high calcium concentrations (see section 2.1.3). In control hearts, VF developed 220±11s (n=6) after initiation of arrhythmogenic perfusion. In hearts excised from rats pre-treated with 20mg/kg allopurinol as previously described, the onset time of VF was 191±17s (n=6). There was, therefore, no evidence of a class I action, although a more detailed investigation is recommended.

(e) Diethyldithiocarbamate (DDC):

Evidence has been reported (Woodward and Zakaria, 1985; Bernier *et al*, 1986) which implicates an arrhythmogenic role for $O_2 \cdot^-$, since SOD reduced the incidence of reperfusion arrhythmias in both of these studies. Consequently, inhibition of endogenous cardiac SOD would be expected to exacerbate arrhythmias initiated by $O_2 \cdot^-$-related mechanisms. This supposition was tested using hearts from rats pre-treated with 1.5g/kg DDC (ip 1 hour), a drug

previously shown by Heikkila and co-workers (1976) to inhibit SOD at this concentration by 81% 1 hour after administration to mice. Hearts were perfused throughout experiments with buffer containing 5.9mM potassium. The low incidence of reperfusion arrhythmias obtained with this concentration of potassium facilitated the observation of any detrimental (proarrhythmic) effects of DDC. The results of these experiments are illustrated in figure 4.3.2.

In control animals, the incidences of VT and VF were 64% and 55% respectively. All hearts from DDC-treated rats underwent episodes of VT, representing a statistically significant increase. The incidence of VF (50%) was statistically similar to the control value, although the duration of VF was longer (19±4s) than that of the control group (8±2s).

To verify the inhibitory action of DDC on SOD, the ferricytochrome c reduction assay was employed (McCord and Fridovich, 1968). Figure 4.3.3 shows the effect of 4 concentrations of SOD (12.5mU, 25.0mU, 37.5mU and 50.0mU) on the rate of ferricytochrome c reduction induced by 2mU of xanthine oxidase in the presence of 50μM xanthine. These experiments were carried out in a cell-free system of 1ml final volume (see section 2.4.2). When administered alone, xanthine oxidase reduced ferricytochrome c at a rate of 0.070±0.001 absorbance units/min. In the presence of the above concentrations of SOD, the reduction rate was lowered to 0.045±0, 0.031±0.009, 0.035±0.004 and 0.024±0.006 absorbance units/min respectively. The inclusion of 0.01mM and 0.10mM DDC (concentrations calculated from the doses administered to the rats) to the reaction mixture reduced

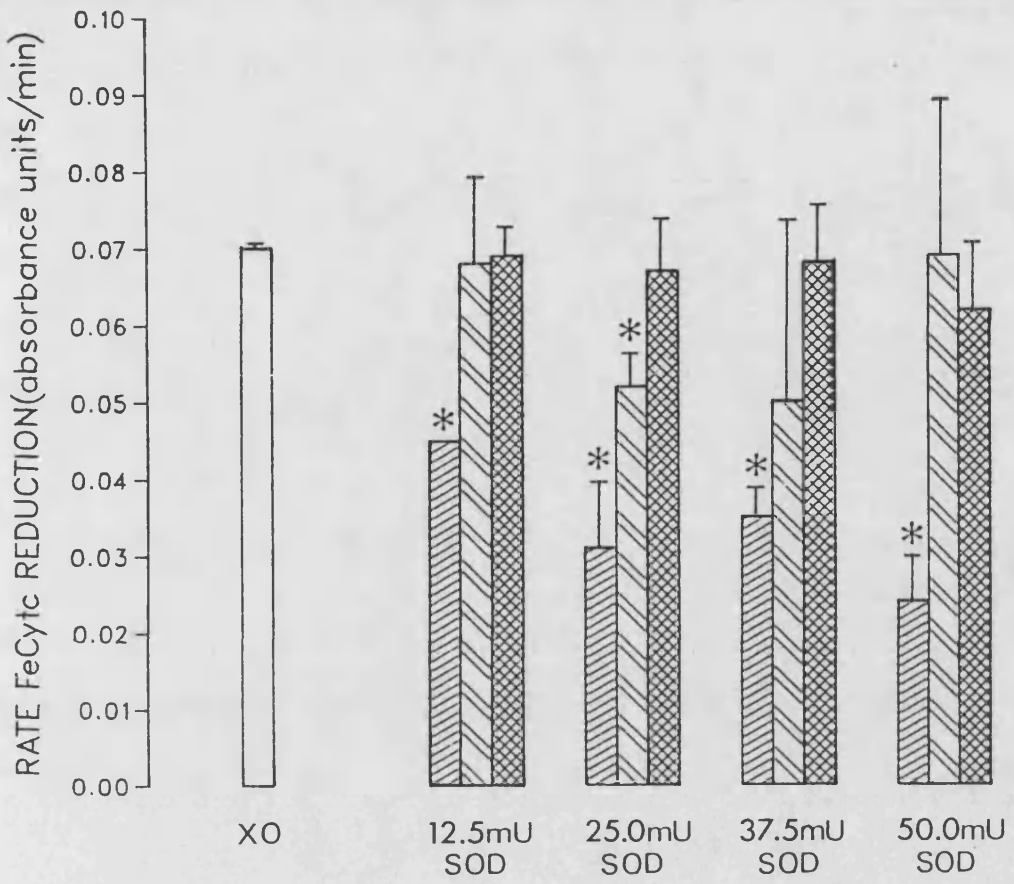


Figure 4.3.3

The rate of ferricytochrome c reduction (absorbance units/min) induced by 2 mU xanthine oxidase (XO, n=3).

Experiments were repeated in the presence of 4 doses of superoxide dismutase (SOD) alone ▨ and in combination with 0.01 mM diethyldithiocarbamate (DDC) ▧ or 0.1 mM DDC ▩ (n = 2 per group).

The mean and standard error of each reduction rate are shown.

*$p \leqslant 0.05$ compared with the XO group.

the action of SOD in a concentration-dependent manner. 0. 1mM DDC abolished the effects of SOD at all concentrations. With 0.01mM DDC, complete inhibition was only observed at the lowest SOD concentration (12.5mU). These results demonstrate that the superoxide radical scavenger, SOD, is inhibited by DDC *in vitro*.

4.3.2 $^{86}Rb^{+}$ Efflux

(a) Desferrioxamine:

Neither concentration of desferrioxamine (0.1mM and 1mM) significantly altered mean peak $^{86}Rb^{+}$ efflux (0.062±0.014 min^{-1} and 0.057±0.006 min^{-1} respectively) or mean efflux area (0.094±0.022 au and 0.096±0.030 au respectively) compared with the control values (0.076±0.010 min^{-1} and 0.133±0.023 au; figure 4.3.4).

(b) 6-OHDA:

Significant reductions of $^{86}Rb^{+}$ efflux were observed in the hearts of 6-OHDA-treated rats, both in terms of mean peak erc (0.044±0.003 min^{-1}) and mean efflux area (0.052±0.014 au). These changes occurred despite an insignificant reduction of VF incidence in these hearts (section 4.3.1(b)).

(c) Hypoxic perfusion:

$^{86}Rb^{+}$ efflux in hearts rendered hypoxic 2 min prior to CAL was unchanged (0.075±0.014 min^{-1} and 0.147±0.058 au) compared with that of the control group (figure 4.3.4). However, hearts subjected to hypoxic perfusion 2 min before reperfusion exhibited significant increases of both efflux parameters. Mean peak erc rose from the

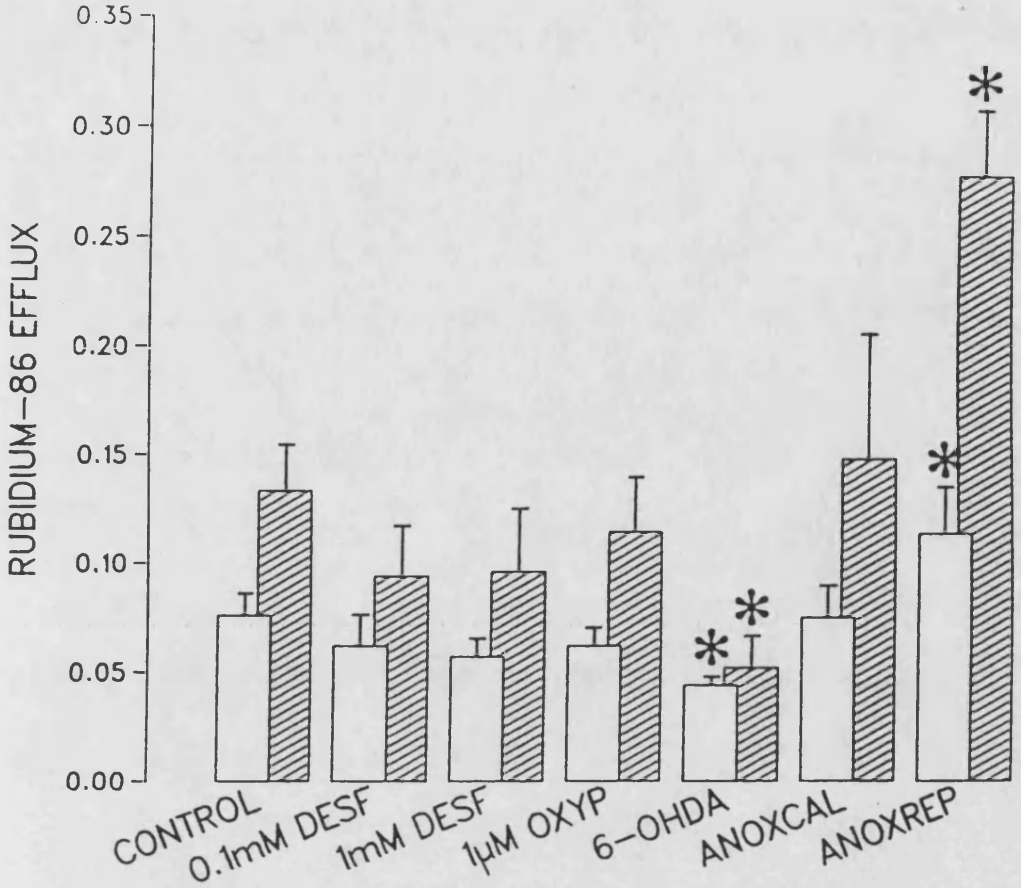


Figure 4.3.4:

Reperfusion-induced $^{86}Rb^{+}$ efflux in control (n=21) and drug-treated hearts.

Mean peak erc (min^{-1}, open bars) and mean efflux area (au, shaded bars) are shown with corresponding standard errors for the first 3 min of reperfusion.

DESF, desferrioxamine (0.1mM and 1mM, n=6 per group); OXYP, oxypurinol (1μM n=7); 6-OHDA, 6-hydroxydopamine (60mg/ml n=6); ANOXCAL, hypoxically perfused from 2 min before CAL (n=5); ANOXREP, hypoxically perfused from 2 min before reperfusion (n=6).

* $p<0.05$ compared with the control group.

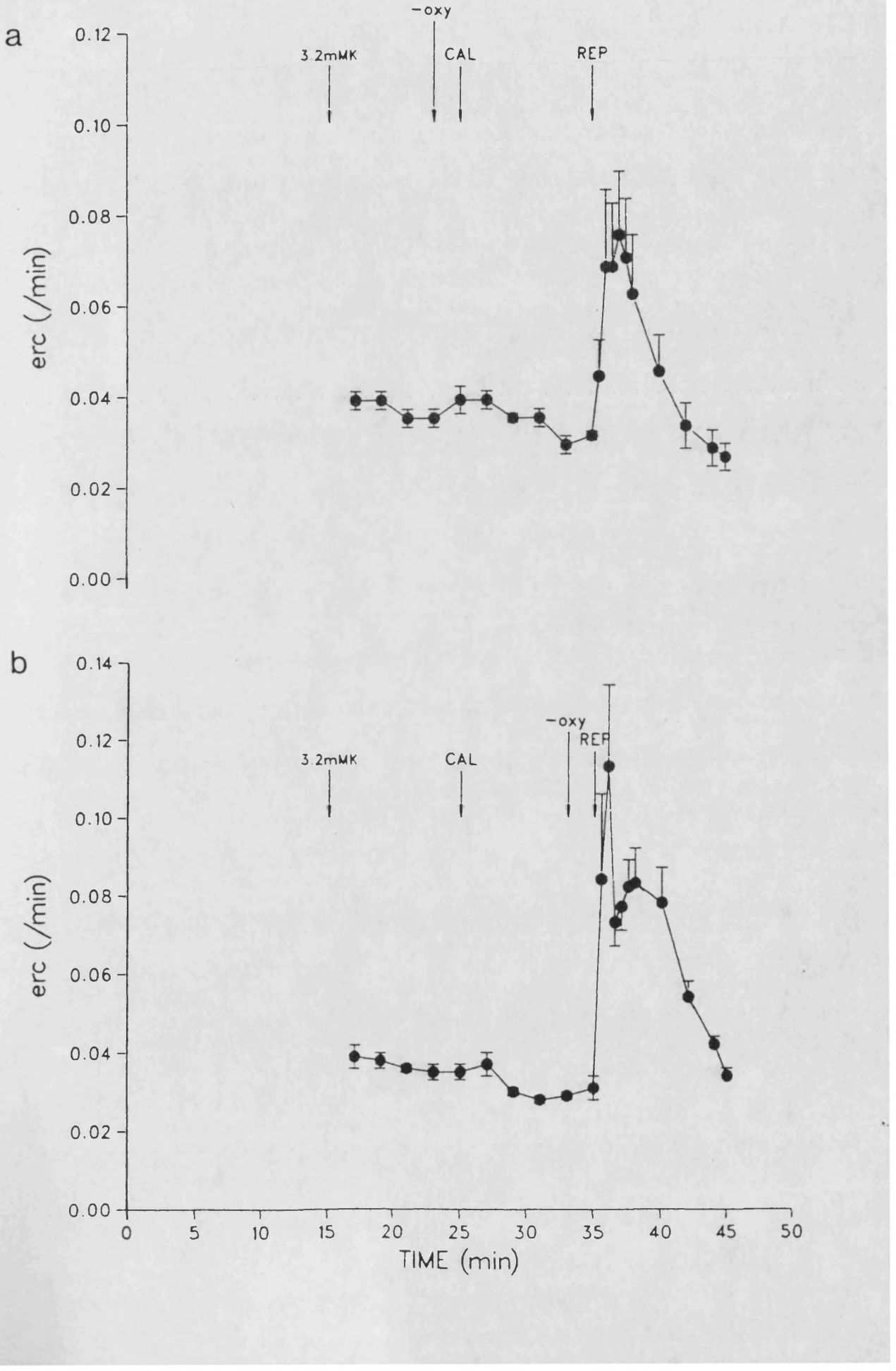
control value of 0.076±0.010 min^{-1} to 0.113±0.021 min^{-1}. Mean efflux area in the control group was 0.133±0.023 au; the corresponding value for the ANOXREP group was 0.276±0.032 au. The ANOXCAL data show that although VF duration was significantly increased, there was no corresponding increase in $^{86}Rb^{+}$ efflux. $^{86}Rb^{+}$ efflux time profiles for both protocols (figure 4.3.5) reveal that hypoxic perfusion, when initiated 2 min before CAL (figure 4.3.5(a)), transiently raised $^{86}Rb^{+}$ erc. This effect was maximal 2-4 min after the start of hypoxic perfusion, but was probably not of sufficient magnitude to add significantly to the post-reperfusion response of the ANOXREP group (figure 4.3.5(b)). It is likely, therefore, that other mechanisms were responsible for the elevation of reperfusion-induced $^{86}Rb^{+}$ efflux in hypoxic hearts. This possibility is discussed in section 4.4.

(d) Allopurinol and oxypurinol:

The substantial reduction of the incidence of VF in hearts from allopurinol-treated rats (section 4.3.1(d)) was not associated with a significant change in reperfusion-induced $^{86}Rb^{+}$ efflux (figure 4.3.6). The absence of an effect on $^{86}Rb^{+}$ efflux suggests a dissociation between this parameter and the antiarrhythmic action of allopurinol. However, the lack of protection against arrhythmias afforded by oxypurinol, and the drug's ineffectiveness at reducing $^{86}Rb^{+}$ efflux (figure 4.3.4), are as would be predicted if a relationship between $^{86}Rb^{+}$ efflux and arrhythmogenicity exists. Furthermore, these negative responses suggest that xanthine oxidase endogenous to rat hearts, should it be present at all, is not an important factor in reperfusion arrhythmias in this model.

Figure 4.3.5:

$^{86}Rb^{+}$ erc values (min^{-1}) in hearts perfused with hypoxic perfusion fluid (a) 2 min before coronary artery ligation (CAL, n=5) or (b) 2 min before reperfusion (REP, n=6).


a
b
−oxy
3.2mMK
CAL
REP
erc (/min)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.14
0
5
10
15
20
25
30
35
40
45
50
TIME (min)

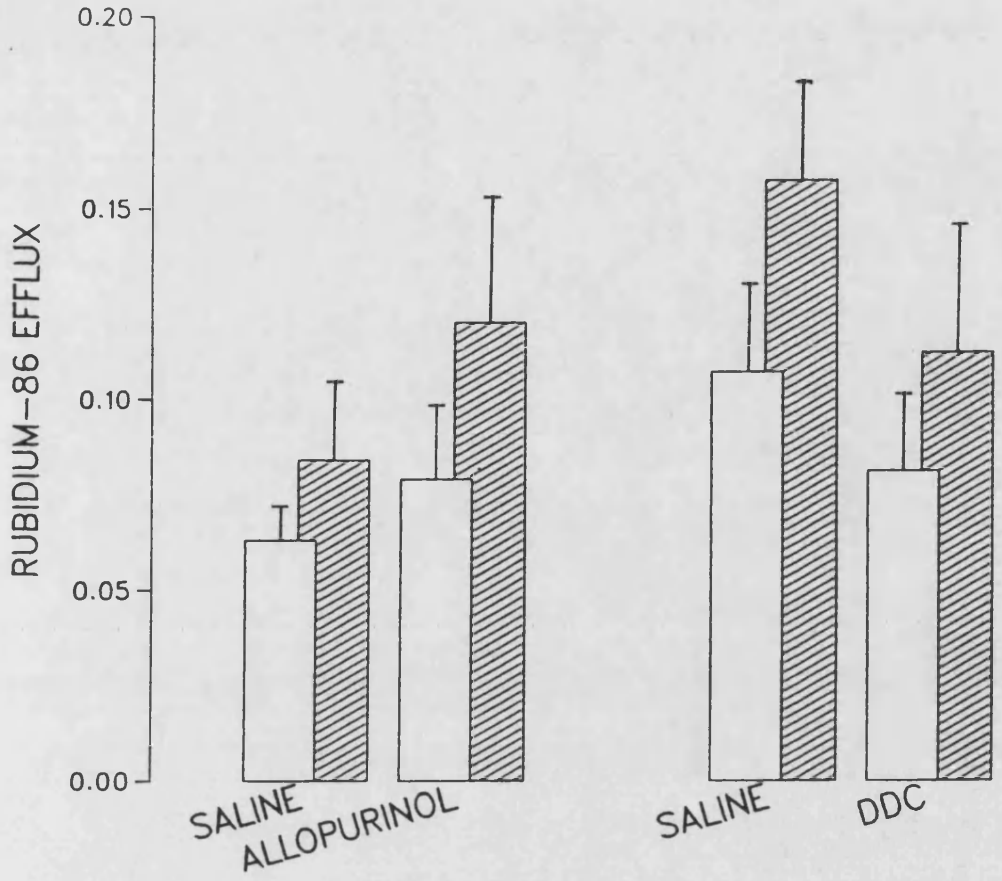


Figure 4.3.6

Reperfusion-induced rubidium-86 efflux in hearts from rats pre-treated with allopurinol (20 mg/kg, ip 48 hours, iv 15 min, n = 8) and diethyldithiocarbamate (DDC, 1.5 g/kg, ip 1 hour, n = 5).

In control groups, 0.9 % w:v saline was administered in place of the drug (allopurinol control, n = 10; DDC control, n = 5). Hearts in DDC control and test groups were perfused with buffer containing 5.9 mM potassium (section 4.3.1 (e)).

Vertical bars represent mean peak erc (min^{-1}, open bars) and mean efflux area (au, shaded bars), with corresponding standard errors (vertical lines), for the first 3 min of reperfusion.

*$p \leqslant 0.05$ compared with the appropriate control group.

(e) Diethyldithiocarbamate (DDC):

Hearts from DDC-treated rats did not exhibit significant changes in either mean peak erc or mean efflux area (figure 4.3.6), despite enduring an increased incidence of VT. The detrimental effect of SOD-inhibition by DDC, whilst increasing the duration of VF, was apparent from this observation alone. Should there be a relationship between the occurrence of arrhythmias and an elevated efflux of $^{86}Rb^{+}$, it would appear from these results that VT is not a major cause or consequence of the latter response. In addition, these data imply that $O_2\cdot^-$ or $O_2\cdot^-$-derived radicals are involved in the genesis of VT, but are not major determinants of VF, although other possible explanations for these results will be discussed later.

4.3.3 Haemodynamic Changes

(a) Desferrioxamine:

Perfusion of hearts with desferrioxamine (0.1mM and 1mM) did not significantly alter HR or dT. However, both concentrations reduced PP (figure 4.3.7), although these responses were not concentration-dependent. 0.01mM desferrioxamine lowered PP by 18$\pm$2mmHg, representing a statistically significant change. This vasodilator action did not reduce the incidence of arrhythmias (section 4.3.1(a)) or alter the reperfusion-induced efflux of $^{86}Rb^{+}$ (section 4.3.2(a)). There was, therefore, no evidence of coronary steal protection in these experiments.

(b) Oxypurinol:

1μM oxypurinol significantly reduced dT by 1.02±0.26g and induced a fall in PP of 13±4mmHg (figure 4.3.7). As with desferrioxamine, the vasodilator response to oxypurinol was not associated with a reduction in arrhythmias or the magnitude of $^{86}Rb^{+}$ efflux.

(c) Hypoxic perfusion:

The main haemodynamic response of hearts to hypoxia was a reduction of PP (figure 4.3.7). Early induction of hypoxia (ANOXCAL) resulted in a substantial, significant fall in PP of 23±4mmHg. This effect was achieved 3-4 min after the initiation of hypoxic perfusion. When induced 2 min before reperfusion (ANOXREP), hypoxia caused PP to fall by 14±2mmHg. In the ANOXCAL group, the reduction of dT (1±0.5g) achieved statistical significance.

The vasodilator response of hearts to hypoxia was again not associated with protection against arrhythmias or accompanied by a reduction of reperfusion-induced $^{86}Rb^{+}$ efflux.

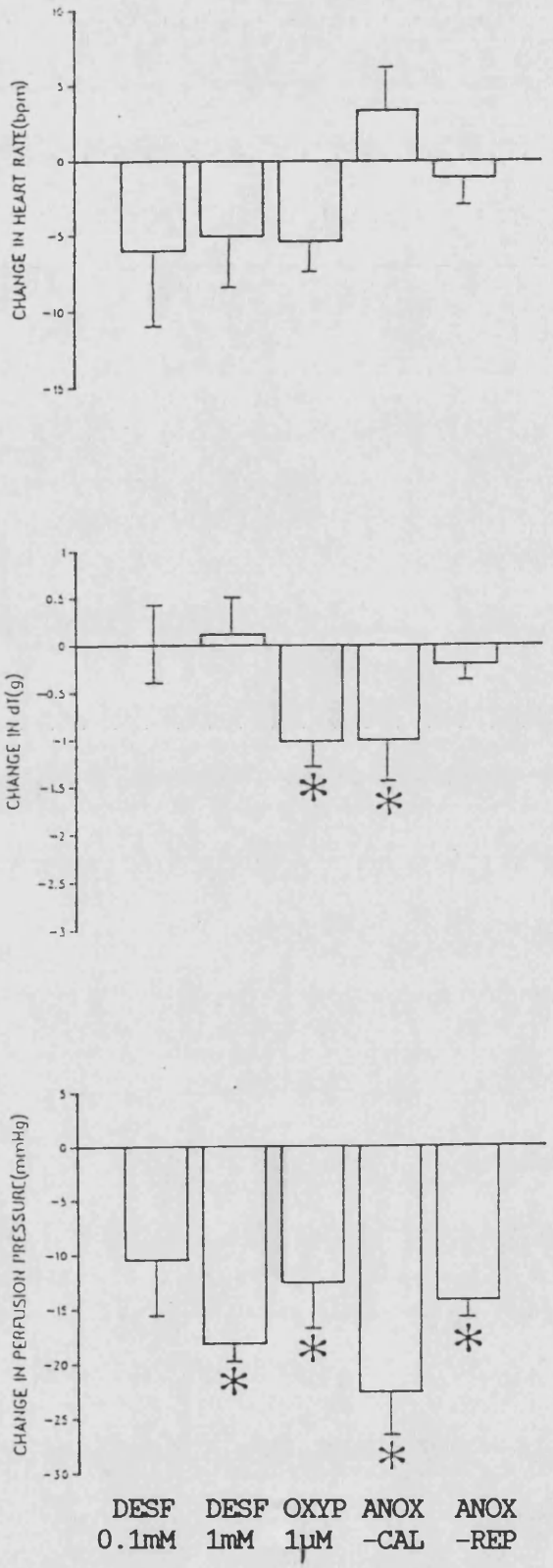


Figure 4.3.7:

Haemodynamic responses of isolated rat hearts to desferrioxamine (DESF; 0.1mM and 1mM,n=10), oxypurinol (OXYP; 1μM, n=13) and hypoxic perfusion from 2 min before CAL (ANOXCAL,n=12) or 2 min before reperfusion (ANOXREP,n=9)

Results are expressed as the mean and standard error of the change following treatment.

* $p<0.05$ compared with the pre-treatment value.
For pre-treatment values, see figure 4.2.3.

4.4 DISCUSSION

Protection against reperfusion-induced arrhythmias was afforded by GSH in accordance with similar observations by other groups (Woodward and Zakaria, 1985; Bernier *et al*, 1986). The antiarrhythmic action of GSH was not concentration-dependent, and this contrasts with the results of Woodward and Zakaria (1985) who achieved a greater reduction in the incidence of VF with increasing concentrations of GSH. However, Bernier and colleagues (1986) demonstrated an inverse relationship between GSH concentration and the incidence of fibrillation. The reason for these equivocalities is not readily apparent although the vasodilator component observed in the present study may be a contributory factor. Whilst Bernier and co-workers (1986) obtained a GSH-mediated increase in coronary flow of approximately 50% during ischaemia, this effect was not discussed. The ability of the GSH/GSH peroxidase system to "scavenge" H_2O_2 and reduce the formation of $OH\cdot$ is now firmly acknowledged, but the contribution of this system to the antiarrhythmic action of GSH in isolated hearts has not been quantified. It is therefore conceivable that GSH exerts multiple effects that act synergistically to reduce the vulnerability of hearts to reperfusion arrhythmias.

The results presented previously show that, whilst the maximum antiarrhythmic protection of GSH was achieved at a concentration of 0.1mM, a reduction of the reperfusion-induced rise in $^{86}Rb^+$ efflux was only observed with 1mM GSH. This complicates the direct correlation between arrhythmia incidence and $^{86}Rb^+$ efflux elevation

in this model. The greatest dilator action of GSH was also obtained with a 1mM concentration and it may be prudent to suggest an association between dilation and the degree of $^{86}Rb^{+}$ efflux. This possibility was investigated further and is detailed in chapter 5.

An important consideration with regard to GSH's anti-free radical action in the model studied is a reliance upon the delivery to the heart of reduced GSH. Any molecule or compound that contains free sulphydryl groups is vulnerable to oxidation to the disulphide (Kosower and Kosower, 1978). The susceptibility of sulphydryl agents to oxidation varies according to the steric stability afforded by adjacent groups on the molecule (B.Swann; personal communication). Gassing solutions of GSH with 95% O_2/5% CO_2 may therefore oxidise GSH to GSSG, although GSH has been reported to react sluggishly with O_2 (Meister and Anderson, 1983). However, it was considered important to determine whether GSH oxidation had occurred during gassing in the experiments described earlier. ^{1}H NMR analysis of freshly prepared solutions of GSH and GSSG (before and after a 1 hour period of gassing) confirmed that little, if any, GSH oxidation had taken place (S.Branch; personal communication).

Ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions are important catalysts in free radical generating reactions in aqueous solutions (Halliwell and Gutteridge, 1984). Although tissue iron levels have been shown to rise in the hearts of dogs during experimental coronary artery occlusion (Aust and White, 1985), and perfusion of isolated rat hearts with the iron chelator desferrioxamine reduced the incidence of reperfusion arrhythmias in the study by Bernier *et al* (1986), a

direct correlation between cardiac iron levels and reperfusion arrhythmogenesis has not been demonstrated. This may be a consequence of the lack of specificity of iron assay systems such as that employed and reported by Aust and White (1985), which did not differentiate between the various iron chelates present in tissues. Ferritin-bound iron constitutes the largest single pool of iron in cells (Aust and White, 1985), and this is sequestered in an inert form. Despite the latter authors' assertion that iron bound to ferritin may be released to participate in oxidation reactions such as those involved in free radical generation, the time-course and extent to which this occurs has again not been demonstrated in ischaemic hearts. The results of the present study, which demonstrated a lack of antiarrhythmic protection and a failure to reduce $^{86}Rb^{+}$ efflux with desferrioxamine, suggest that iron does not participate in the generation of arrhythmias during early reperfusion, and this finding concurs with the results of Woodward and Zakaria (1985). Alternatively, it is possible that desferrioxamine does not chelate iron sufficiently to prevent its catalytic role in free radical production. This seems unlikely, since desferrioxamine has proved effective in ameliorating the symptoms of other disease states associated with iron overload (Halliwell and Gutteridge, 1985). A final possible explanation may be that iron-catalysed free radical generating reactions only contribute to the pathogenesis of reperfusion damage in the heart at a later stage. Such reactions are thought to be the underlying cause of lipid peroxidation, a degradative process manifested as irreversible myocardial damage and ultimately myocardial infarction (Meerson *et al*, 1982). Aust and White (1985) postulated that the

initial release of tissue iron facilitates the release of additional iron which participates in processes of tissue destruction. It is the authors' belief that desferrioxamine cannot prevent early iron release but can alleviate subsequent iron-induced tissue damage. In the light of the current results, further work is clearly required to elucidate the role of iron in putative free radical-mediated reactions resulting in arrhythmogenesis. This work must entail specific measurements of tissue iron over a longer time-course than that employed in the present experiments. The discrepency concerning the antiarrhythmic action of desferrioxamine demonstrated by Bernier *et al* (1986) cannot, as yet, be explained, although detailed comparisons of experimental conditions and the commercial reagents employed may expose the critical variant. The cause of this disparity may, in itself, help to elucidate the arrhythmogenic mechanisms of reperfusion.

The degree to which myocardial catecholamine depletion protects against reperfusion arrhythmogenesis is not clear from experiments using 6-OHDA-treated animals. The lower incidence of arrhythmias observed in this group did not significantly differ from control values, although the onset of VT was delayed. The reperfusion-induced rise in $^{86}Rb^{+}$ efflux was significantly attenuated however. The reduction of arrhythmogenesis in this group, despite its lack of statistical validity, implies that catecholamines are involved to some extent in this response. Whether the production of free radicals is associated with this action cannot be determined from the data described, although

Woodward and Zakaria have demonstrated significantly lower free radical production in hearts from 6-OHDA-treated rats (unpublished data, cited by Woodward and Manning, 1987). Sheridan *et al* (1980) were also able to reduce the incidence of reperfusion arrhythmias in cats following 6-OHDA treatment, but ascribed this effect to a reduction of adrenergic receptor stimulation. The protective action of catecholamine depletion may also be of metabolic origin. Daugherty (1981) observed a significantly higher myocardial glycogen level in hearts from 6-OHDA-treated rats subjected to coronary ligation. Preservation of cardiac high energy phosphates through maintained or elevated levels of substrates such as glycogen is undoubtedly of importance in maintaining active systems, including those resposnible for cellular ion homeostasis. The sustained control of $^{86}Rb^{+}$ efflux following reperfusion in catecholamine depleted hearts may therefore be closely related to the energy status of the myocardium, although further studies are required to substantiate this hypothesis.

Myocardial ischaemia has been described as a "priming" period for the production of oxygen radicals upon reperfusion. McCord (1984) has suggested that ischaemia-induced changes result in xanthine oxidase-derived radical generation (see below). The availability of oxygen is another, seemingly obvious, prerequisite for reperfusion-induced oxygen radical production. However, reducing this availability, by perfusing hearts with hypoxic buffer 2 min prior to reperfusion, did not afford any protection to hearts in terms of arrhythmias or $^{86}Rb^{+}$ efflux. When hypoxic perfusion was initiated 2 min prior to the ischaemic interval, some

antiarrhythmic protection was apparent; although the incidences of arrhythmias were unchanged, there was a tendancy for arrhythmic episodes to be delayed. These findings implicate the importance of oxygen availability during ischaemia (rather than during reperfusion) to the mechanisms responsible for reperfusion arrhythmogenesis. Hypoxia-induced lowering of intracellular ATP concentrations and/or a reduction of the affinity of active systems for ATP during hypoxia (Allen and Orchard, 1987) may perturb ion homeostatic mechanisms to a degree that affects action potential characteristics. Noma (1983) has described a calcium-independent, ATP-regulated potassium channel in cardiac muscle that increases outward potassium currents at low (<0.2mM) ATP concentrations. In normal cardiac cells, the intracellular ATP concentration has been reported to be 3-4mM (Khairallah and Mommaerts, 1953), whereas during hypoxia, this concentration falls by some 90% (Grinwald *et al*, 1980). Noma (1983) suggested that activation of this potassium channel may reduce contractility (as observed during hypoxia; Allen and Orchard, 1987) and so preserve ATP levels by reducing ATP consumption. The results of the present study, which identified an increased efflux of $^{86}Rb^{+}$ (above control values) following reperfusion after 2 min of hypoxic perfusion, might be explained by the existence of a potassium channel regulated by intracellular ATP concentrations. However, if this was the case, why was $^{86}Rb^{+}$ efflux not elevated above control levels upon reperfusion after 12 min of hypoxic perfusion? One possible explanation involves the dilator effect of hypoxia. Prolonged hypoxic perfusion (12 min) reduced perfusion pressure almost two-fold compared with the shorter (2 min) period of hypoxia. The former response was likely to be the

result of metabolic vasodilation in both the occluded (hypoxic and ischaemic) zone and the non-occluded (hypoxic only) zone, whereas in the latter situation (2 min period of hypoxia), only the occluded zone is expected to have undergone metabolic (ischaemic only) dilation up to the time at which hypoxic perfusion was initiated. Thus, when hypoxia was induced before CAL, dilation of non-ischaemic vessels would be greater at the time of reperfusion than that seen when hypoxia was induced only 2 min before reperfusion. In effect, subjecting hearts to hypoxic conditions before the onset of ischaemia would reduce the relative difference between the diameter of vessels in the ischaemic and non-ischaemic vascular beds. This, through coronary steal, would reduce the rate of reperfusion and washout from the ischaemic zone.

None of the above hypotheses implicate the involvement of oxygen radicals in the arrhythmic and $^{86}Rb^{+}$ efflux responses obtained using hypoxia protocols. It would seem straightforward to assume that by reducing the availability of oxygen, the generation of oxygen radicals would be limited. However, several other factors require to be considered. Firstly, was oxygen availability sufficiently restricted? Guarnieri and colleagues (1980) employed an identical perfusion system to that used in the current study and found perfusate oxygen tensions for aerobically gassed (95% O_2/5% CO_2) and hypoxically gassed (95% N_2/5% CO_2) buffer solutions to be >600mmHg and <6mmHg respectively. In our laboratory, measurements using a non-airtight oxygen electrode showed hypoxic buffer to contain less than 10% of the oxygen content of aerobic buffer after passing through the heart perfusion apparatus. Whilst this

demonstrates that nitrogen gassing severely reduced perfusate oxygen levels, it is possible that sufficient oxygen remained to allow oxygen radical generation. Furthermore, oxygen is approximately 8 times more soluble in organic solvents than in water (Halliwell and Gutteridge, 1985). The hydrophobic interior of biological membranes is thought to have the consistency of a light oil and it is therefore likely that oxygen will be retained in this compartment for some time, even under conditions of low oxygen tension. Finally, whilst hypoxic conditions may reduce the amount of oxygen radical production, this beneficial effect may be compromised by a reduced activity of cellular anti-free radical defence systems, as has been demonstrated and reviewed by Guarnieri and co-workers (1980). Despite the above considerations, which require further experimental support, the hypoxia data presented earlier tend to indicate that oxygen radical generation during reperfusion of regionally ischaemic hearts is not a major factor in the development of reperfusion arrhythmias. It is suggested that metabolic changes within the heart under hypoxic conditions may ultimately affect the ionic homeostasis of excitable tissue and in this way may enhance the vulnerability of the heart to ventricular rhythm disturbances. The possibility that $^{86}Rb^{+}$ efflux under these conditions is channel-regulated should be investigated further.

Hearts from DDC-treated rats underwent more episodes of tachycardia and longer durations of fibrillation than those from saline-treated controls, and this indicates a pro-arrhythmic role for $O_2\cdot^-$. However, DDC is not specific for SOD enzymes. Heikkila and associates (1976) reported the use of DDC to inhibit aldehyde

dehydrogenase in liver and dopamine beta-hydroxylase in adrenergic nerves. The pro-drug of DDC, disulfiram, is used in alcohol abuse therapy and exerts many potentially lethal side-effects including VT and acute congestive heart failure (see Bowman and Rand, 1980). Although the mechanisms involved in these actions are not known, Heikkila *et al* (1976) have shown disufiram to be relatively ineffective in inhibiting SOD. The possibility that the adverse tachycardia observed in the present investigation was similarly unrelated to SOD inhibition must preclude the assertion that enhanced $O_2 \cdot^-$ production (resulting from reduced SOD-catalysed dismutation) was directly responsible for this effect despite evidence that DDC did indeed inhibit the enzyme in a cell-free system. Future work requires the measurement of myocardial SOD activity in hearts from DDC-treated animals to enable a direct comparison between SOD inhibition and the incidence of arrhythmias.

Finally, allopurinol has been shown to be antiarrhythmic in the isolated rat heart model via mechanisms that are apparently unrelated to the inhibition of xanthine oxidase. This was demonstrated by the lack of antiarrhythmic protection afforded by the active metabolite, oxypurinol. The mode of action of allopurinol has not been investigated in the present study, but DeWall and colleagues (1971) showed allopurinol treatment to increase myocardial contractility and cardiac output, reverse ischaemia-induced S-T segment elevation and exhibit prolonged antiarrhythmic effects in anaesthetised dogs and sheep. Allopurinol also enhanced purine salvage and consequently maintained myocardial ATP levels. It is also possible that allopurinol exerts a direct

action on myocardial conducting fibres, although the results of preliminary experiments using an "arrhythmogenic" buffer solution suggested that the drug has no class I antiarrhythmic action. Additional studies are required to elucidate allopurinol's mode of action which, in the light of the results presented here, should not be confined to its role of xanthine oxidase inhibition.

In conclusion, several anti-free radical interventions have proved ineffective at preventing the genesis of reperfusion-induced arrhythmias in the isolated rat heart model. With respect to the 6-OHDA and allopurinol data, there would not appear to be a consistent relationship between reperfusion arrhythmias and the magnitude of the accompanying $^{86}Rb^{+}$ efflux, although it is interesting to note that both of these drugs have been associated with the maintainance of energy/substrate levels in the myocardium. GSH was the only free radical scavenging agent that afforded protection against arrhythmias, but these responses were complicated by substantial falls in perfusion pressure following addition of the drug. The importance of this dilator component is considered in chapter 5.

CHAPTER 5

5.1 INTRODUCTION: THE ANTIARRHYTHMIC ACTION OF GLUTATHIONE - CORONARY STEAL OR FREE RADICAL SCAVENGING?

Reduced glutathione (GSH) protects isolated rat hearts against the development of arrhythmias and reduces the efflux of $^{86}Rb^{+}$ following coronary artery reperfusion (chapter 4). Interpretation of these results is complicated by the observation that GSH exerts a pronounced dilator action on the coronary circulation. The mechanism involved in this response has not been reported. Dilation of coronary vessels in the non-ischaemic area of myocardium may act to divert coronary flow from vessels serving the ischaemic zone, thereby reducing the rate at which ischaemic tissue is reperfused (the "coronary steal" effect). Since a reduction of the rate of reperfusion reduces the incidence of arrhythmias in this model (Zakaria, 1985), it is possible that the antiarrhythmic effect of GSH is not related to the drug's anti-free radical action. Whilst administration of GSH to isolated rat hearts 2 min before reperfusion has also been shown to reduce arrhythmias and $^{86}Rb^{+}$ efflux (Zakaria, 1985), the possibility still remains that coronary dilation could have occurred within this 2 min period sufficient to allow coronary steal.

Free radical related cellular damage is accompanied by a reduction of tissue GSH levels (Guarnieri *et al*, 1979; Ferrari *et al*, 1985), is accentuated by GSH depletion (Barsacchi *et al*, 1984; Harlan *et al*, 1984) and is attenuated by enhancement of intracellular GSH concentrations (Tsan *et al*, 1985). Protection against reperfusion arrhythmias by GSH has been demonstrated in isolated hearts

(Woodward and Zakaria, 1985; Bernier *et al*, 1986) and has been ascribed to the free radical scavenging property of GSH, but little, if any, emphasis has been made on the dilator action and possible anti-ischaemic effects of this drug. Described below are the results of experiments designed to establish: (a) whether other sulphydryl-containing compounds exert similar responses in isolated hearts to those of GSH, and (b) if vasodilator drugs reproduce these effects.

The sulphydryl compounds employed in this study were d-penicillamine (PSH) and dithiothreitol (DTT). PSH is a copper chelating agent used in the treatment of Wilson's disease (an inherited disease characterised by low levels of the body's endogenous copper chelating agent caeruloplasmin) (Halliwell and Gutteridge, 1985) and as an anti-rheumatic drug (Roberts and Robinson, 1985). The mode of action of PSH in rheumatoid arthritis is unknown, although recent evidence (Roberts and Robinson, 1985) suggests that copper-PSH complexes exhibit SOD-like properties. DTT, a disulphydryl compound, is commonly used in experimental studies to reduce disulphide groups to free thiols (Bellomo *et al*, 1983; Thor *et al*, 1985; Nicotera *et al*, 1985) and as such is a useful tool in investigations of the importance of sulphydryl groups to cellular processes.

Vasodilator drugs are used clinically in the treatment of hypertension and congestive heart failure. In myocardial ischaemia, certain vasodilators have been shown to reduce ventricular filling and intramural coronary artery compression and so facilitate

increased collateral blood flow (Elfellah and Ogilvie, 1985). In the present study, two vasodilator drugs, sodium nitroprusside (NITRO) and hydralazine (HYD), were used in an attempt to reproduce the degree of coronary dilation observed with GSH. Their concentrations were derived from preliminary dose-response studies in which reductions in PP, similar to those achieved with GSH, were obtained without any observable adverse effects on HR and dT.

5.2 RESULTS: RESPONSES OF ISOLATED HEARTS TO SULPHYDRYL COMPOUNDS AND VASODILATOR DRUGS

5.2.1 Arrhythmias

(a) Sulphydryl compounds: PSH and DTT:

Neither PSH (0.1mM and 1mM) or DTT (0.1mM and 1mM) significantly affected the incidences of VT or VF upon reperfusion (figure 5.2.1). The onset and duration of arrhythmias and the number of PVC's were similarly unaffected (table 5.2.1).

(b) Vasodilator drugs: NITRO and HYD:

NITRO (0.01μM and 0.1μM) was without effect on the incidence of arrhythmias in reperfused hearts (figure 5.2.1). However, 0.01μM NITRO significantly prolonged the duration of VF from the control value of 75±13s to 148±14s (table 5.2.1). The incidences of VT and VF in hearts perfused with HYD were also statistically unaffected (figure 5.2.1), although this drug delayed the onset of both types of arrhythmia (table 5.2.1). The first episode of VT occurred after 36±14s, a significant increase compared with 6±1s in control hearts. VF was observed 36±7s after reperfusion, almost three times the value for the control group (13±2s). HYD also reduced the number of PVC's from 199±30 (control) to 72±25. Antiarrhythmic protection with vasodilator drugs was therefore minimal despite accompanying reductions of PP (section 5.2.3).

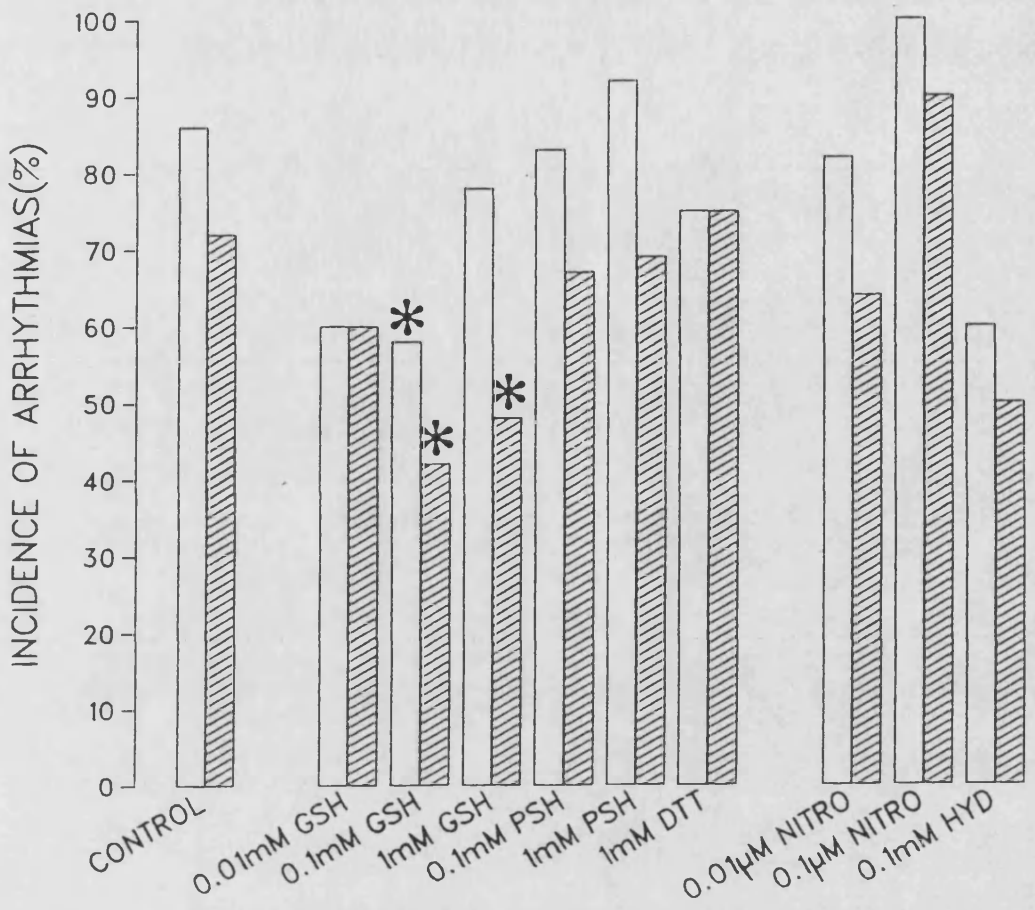


Figure 5.2.1:

The percentage incidence of VT (open bars) and VF (shaded bars) following reperfusion in control hearts (n=43) and hearts perfused with sulphydryl compounds or vasodilator drugs.

GSH, glutathione (0.01mM, 0.1mM, 1mM n=10, 12, 27 respectively); PSH, d-penicillamine (0.1mM n=12, 1mM n=13); DTT, dithiothreitol (1mM n=12); NITRO, sodium nitroprusside (0.01μM n=11, 0.1μM n=10); HYD, hydralazine (0.1mM n=10).

* $p<0.05$ compared with the control group.

		VT			VF			PVC's
	n	n	ONSET (sec)	DURATION (sec)	n	ONSET (sec)	DURATION (sec)	
CONTROL	43	37	6± 1	16± 3	31	13± 2	75±13	199±30
PSH:								
10^{-4}M	12	10	8± 5	11± 3	8	17± 8	60±18	121±32
10^{-3}M	13	12	14± 5	11± 3	9	11± 2	25± 8	156±43
DTT:								
10^{-3}M	12	9	18± 2	17± 4	9	17± 3	81±23	214±61
NITRO:								
10^{-8}M	11	9	18± 9	12± 6	7	10± 2	148±14*	125±57
10^{-7}M	10	10	5± 2	6± 2	9	18± 5	113±20	157±26
HYD:								
10^{-4}M	10	6	36±14*	7± 2	5	36± 7*	111±22	72±25*

Table 5.2.1:

The onset and duration of VT and VF and the number of PVC's during the first 3 min of reperfusion in control hearts and hearts perfused with sulphydryl compounds or vasodilator drugs.

PSH, d-penicillamine; DTT, dithiothreitol; NITRO, sodium nitroprusside; HYD, hydralazine.

* $p<0.05$ compared with the control value.

5.2.2 $^{86}Rb^{+}$ Efflux

(a) Sulphydryl compounds: PSH and DTT:

Attenuation of the reperfusion-induced efflux of $^{86}Rb^{+}$ by 1mM GSH (chapter 4 and figure 5.2.2) was also observed in hearts perfused with PSH (figure 5.2.2). 0.1mM PSH reduced the control mean peak erc value (0.076±0.010 min^{-1}) to 0.037±0.003 min^{-1} and the control efflux area (0.133±0.023 au) to 0.037±0.009 au. Corresponding significant reductions with 1mM PSH were 0.041±0.002 min^{-1} and 0.026±0.009 au. Unlike GSH however, the low efflux values seen with PSH did not correspond with an antiarrhythmic action.

Hearts perfused with 0.1mM DTT exhibited mean peak erc and mean efflux area values that were not significantly different to those of the control group, whereas 1mM DTT significantly reduced mean peak erc to 0.049±0.006 min^{-1}, but was without effect on mean efflux area (figure 5.2.2).

(b) Vasodilator drugs: NITRO and HYD:

Both vasodilator drugs significantly attenuated the reperfusion-induced rise of $^{86}Rb^{+}$ efflux (figure 5.2.3). 0.1μM NITRO lowered mean peak erc to a value of 0.042±0.004 min^{-1} and mean efflux area to 0.055±0.013 au. 0.01μM NITRO decreased mean peak erc and mean efflux area to values of 0.046±0.008 min^{-1} and 0.044±0.013 au respectively. There appeared to be no positive correlation between arrhythmia incidence and $^{86}Rb^{+}$ efflux following reperfusion of NITRO-perfused hearts (figure 5.2.3). Whilst the incidences of VT and VF were reduced in the HYD group, these changes were not

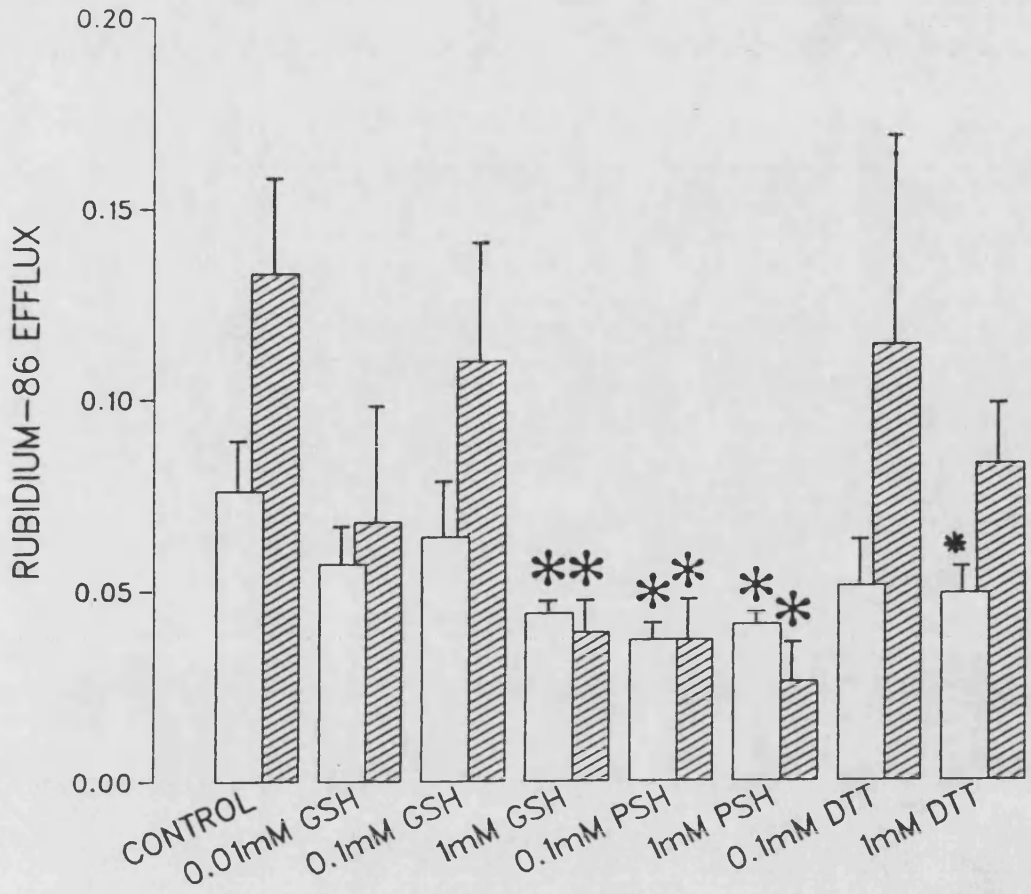


Figure 5.2.2

Reperfusion-induced rubidium-86 efflux in control hearts (n = 21) and hearts perfused with sulphydryl compounds.

Vertical bars represent mean peak erc (min^{-1}, open bars) and mean efflux area (au, shaded bars), with corresponding standard errors (vertical lines), for the first 3 min of reperfusion.

GSH, glutathione (0.01 mM, 0.1 mM, 1 mM, n = 6, 5 and 21 respectively); PSH, d-penicillamine (0.1 mM, n = 6; 1 mM, n = 6); DTT, dithiothreitol (0.1 mM, n = 6; 1 mM, n = 6).

* $p \leq 0.05$ compared with the control group.

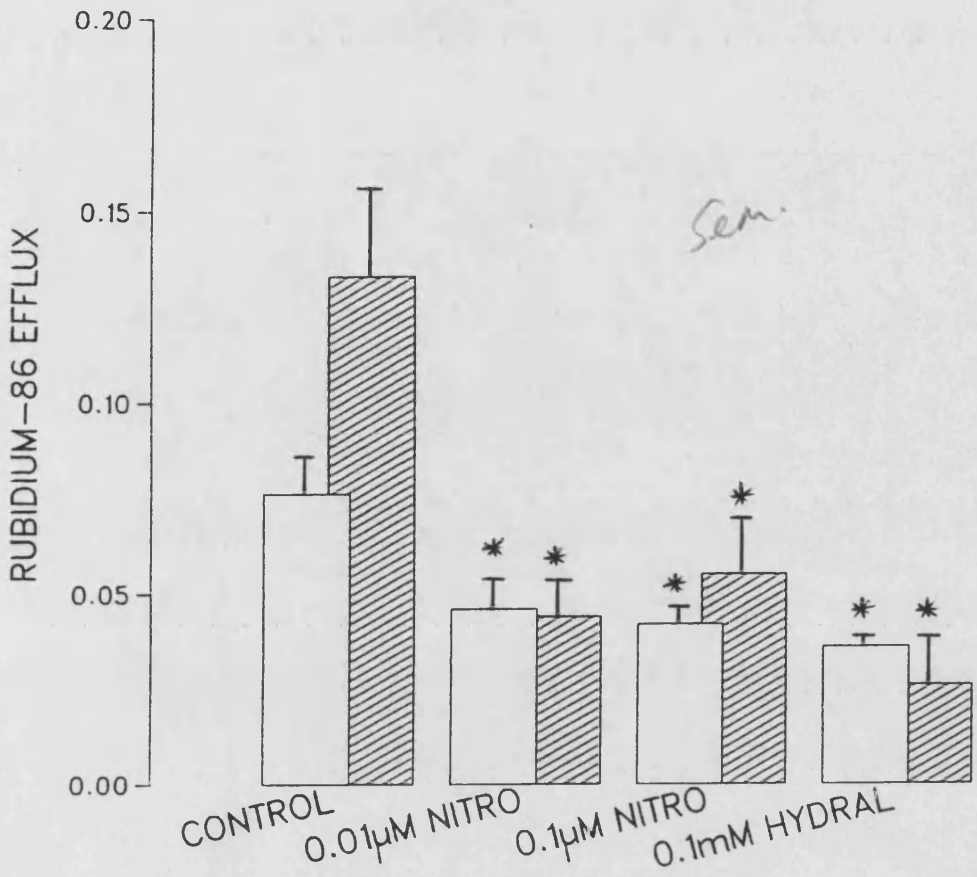


Figure 5.2.3

Reperfusion-induced rubidium-86 efflux in control hearts (n = 21) and hearts perfused with vasodilator drugs.

Vertical bars represent mean peak erc (min^{-1}, open bars) and mean efflux area (au, shaded bars), with corresponding standard errors, for the first 3 min of reperfusion.

NITRO, sodium nitroprusside (0.01 μM, n = 5; 0.1 μM, n = 8);HYD, hydralazine (0.1 mM, n = 5).

* $p \leq 0.05$ compared with the control group.

statistically significant. The attenuations of mean peak erc (0.036 ± 0.002 min^{-1}) and mean efflux area (0.026 ± 0.011 au) were significant however, demonstrating further a dissociation between arrhythmogenesis and $^{86}Rb^{+}$ efflux.

5.2.3 Haemodynamic Changes

(a) Sulphydryl compounds: PSH and DTT:

In chapter 4, GSH was shown to produce a pronounced, concentration-dependent dilator response in isolated hearts. PSH and DTT produced similar reductions of PP to that seen with 0.1mM GSH (figure 5.2.4). 0.01mM PSH and 1mM PSH lowered PP by 16 ± 3mmHg and 25 ± 4mmHg respectively. These changes were statistically significant. 0.1mM and 1mM DTT induced respective reductions of 18 ± 4mmHg and 15 ± 3mmHg, both of which were also significant dilatations.

Both drugs exhibited a bradycardic action. 0.1mM PSH caused HR to fall significantly by 10 ± 3 bpm and a significant drop of 11 ± 4 bpm was observed with 1mM PSH. The effects of DTT on HR were more pronounced. At a concentration of 0.1mM, DTT lowered the rate by 23 ± 2 bpm whilst 1mM DTT induced a fall of 27 ± 4 bpm (figure 5.2.4).

The depressive action of these drugs was further illustrated by their effects on dT. Significant reductions of dT of 0.80 ± 0.32g and 1.14 ± 0.29g were obtained with 0.1mM and 1mM PSH respectively, while 0.1mM and 1mM DTT caused contractility to fall by 0.67 ± 0.20g and 0.80 ± 0.35g respectively. These changes were similar to those seen with 0.1mM GSH (-0.61 ± 0.36g) and 1mM GSH (-0.91 ± 0.28g) (figure

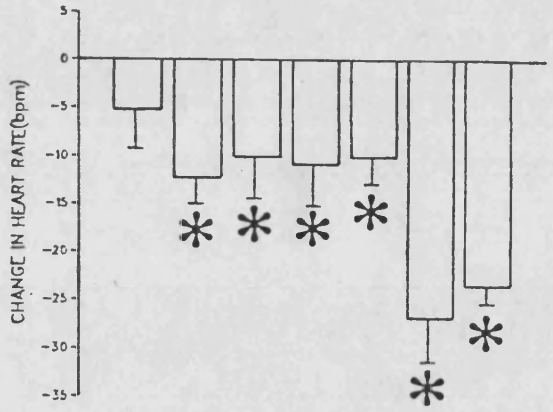
Figure 5.2.4:

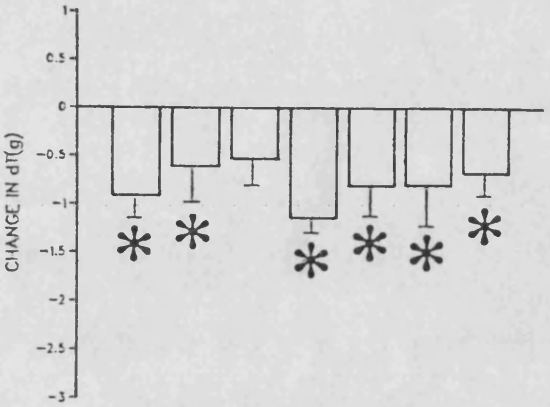
Haemodynamic responses of isolated rat hearts to sulphydryl compounds.

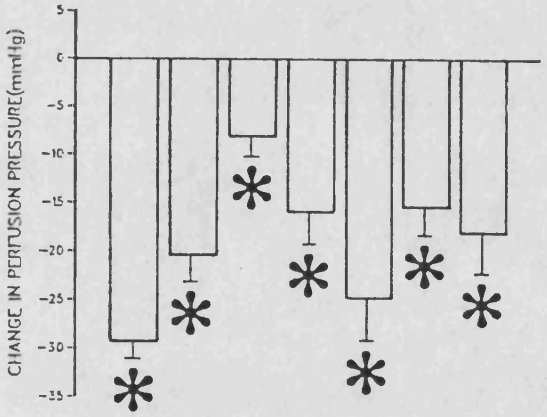
Results are expressed as the mean and standard error of the change observed following drug perfusion.

GSH, glutathione; PSH, d-penicillamine; DTT, dithiothreitol.

* $p<0.05$ compared with the pre-treatment value.
For mean pre-treatment values, see figure 4.2.3.







concentration (M)	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-3}	10^{-4}
n =	27	12	10	13	12	12	6
	GSH			PSH		DTT	

5.2.4).

In summary, all three sulphydryl compounds (GSH, PSH and DTT) produced coronary dilatation, and their effects on dT were similar. DTT was peculiar in its bradycardic action which was more pronounced than that seen with GSH and PSH.

(b) Vasodilator drugs: NITRO and HYD:
0.1mM HYD exerted a pronounced dilator action (PP fell by 28±7mmHg), similar to that produced by GSH, which was accompanied by insignificant changes in HR and dT. 0.01μM NITRO also produced a significant drop of PP of 12±2mmHg, but only at a concentration of 0.1μM NITRO was the dilation observed (17±4mmHg) of similar magnitude to that induced by GSH. However, at this concentration, dT also fell by 1.12±0.31g (figure 5.2.4).

Despite HYD's ability to reproduce the haemodynamic actions of GSH, the vasodilator was unable to protect hearts against arrhythmias. These results therefore support the hypothesis that the antiarrhythmic effects of GSH were not solely related to its dilator action.

Coronary dilation responses to GSH, PSH, DTT, NITRO and HYD were consistent with reductions of the elevation of $^{86}Rb^{+}$ efflux on reperfusion, although the dilation produced by DTT (which was of an approximate magnitude to that seen with NITRO) was only accompanied by a lowering of mean peak erc; mean efflux area was unaffected.

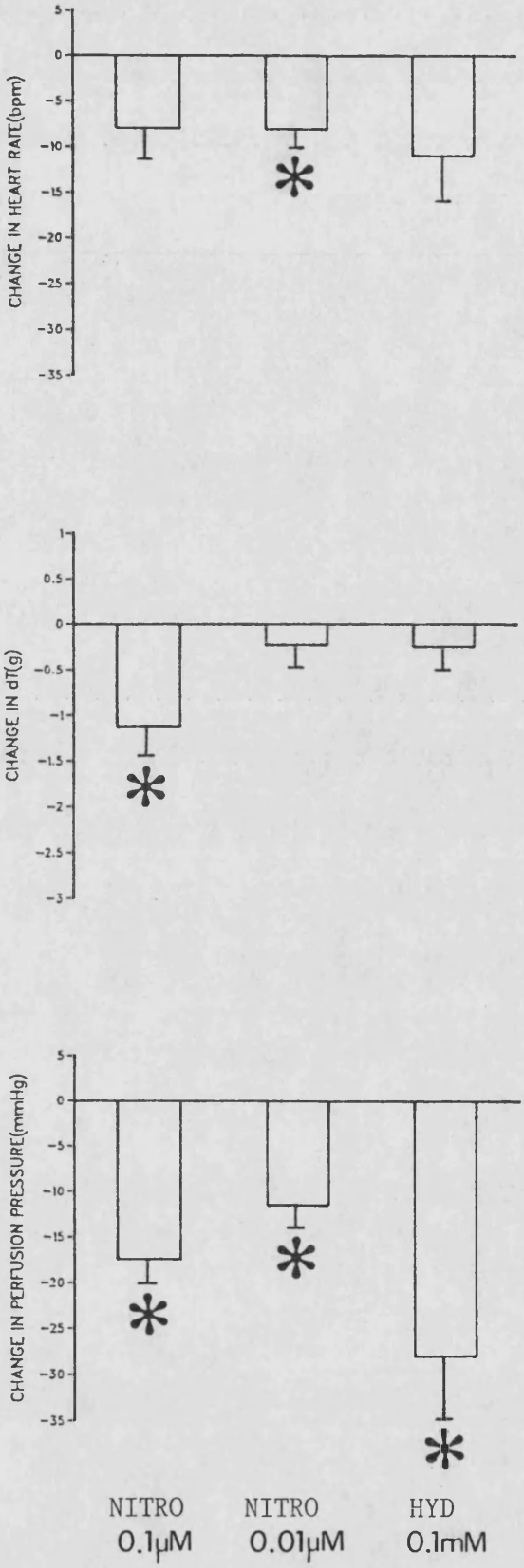
Figure 5.2.5:

Haemodynamic responses of isolated rat hearts to vasodilator drugs.

Results are expressed as the mean and standard error of the change observed following drug perfusion.

NITRO, sodium nitroprusside (0.01μM, n=11; 0.10μM, n=10);
HYD, hydralazine (0.10mM, n=10).

* $p<0.05$ compared with the pre-treatment value.
For mean pre-treatment values, see figure 4.2.3.


CHANGE IN HEART RATE(bpm)
5
0
-5
-10
-15
-20
-25
-30
-35
CHANGE IN dT(g)
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
CHANGE IN PERFUSION PRESSURE(mmHg)
5
0
-5
-10
-15
-20
-25
-30
-35
NITRO 0.1μM
NITRO 0.01μM
HYD 0.1mM

5.3 DISCUSSION

The overall effects of GSH on the haemodynamic functions of isolated rat hearts were depressive and manifested mainly as a marked and concentration-dependent reduction of perfusion pressure. Similar responses were obtained with the two other sulphydryl compounds studied, PSH and DTT. These also reduced perfusion pressure by a similar magnitude to that observed with GSH, although clear dose-response relationships were not demonstrated. Preliminary experiments using the vasodilator drugs sodium nitroprusside and hydralazine produced dose-related reductions in perfusion pressure which, at the concentrations used, were similar in effect to GSH.

Since all drugs were perfused through hearts before coronary ligation, it was conceivable that coronary steal protection against arrhythmias might occur. However, the results presented in this chapter do not indicate that this was the case. Despite the ability of all drugs tested to evoke substantial and similar reductions of perfusion pressure, only GSH was shown to be antiarrhythmic, suggesting that this drug has alternative actions. Whilst there are a multitude of cellular processes that are regulated by thiol-disulphide redox reactions, a factor which complicates interpretation of the effects of exogenous sulphydryl drugs, two other important points should be considered. Firstly, PSH and DTT are not substrates for glutathione peroxidase (Jones *et al*, 1983; B.Swann, personal communication) and cannot, therefore, participate

in the breakdown of H_2O_2. Secondly, the high water solubility of GSH, PSH and DTT suggest that these compounds cannot cross cell membranes in the absence of specific uptake mechanisms. No such mechanisms have been described for PSH and DTT. However, Meister and Anderson (1983) have reviewed evidence indicating that GSH uptake may occur. Extracellular GSH is broken down to gamma-glutamyl amino acids by an enzyme, gamma-glutamyl transpeptidase, located on the outer surface of the plasma membrane. The amino acid substrate for this enzyme is cystine and the product of the reaction, gamma-glutamyl cystine, is transported into the cell where it becomes a substrate for a transhydrogenation reaction resulting in the formation of the oxidised (disulphide) form of cystine, cysteine. The intracellular enzyme gamma-glutamyl cysteine synthetase uses cysteine and glutamine in an ATP-requiring reaction to produce gamma-glutamyl cysteine. A second energy-requiring reaction, catalysed by GSH synthetase, generates GSH from gamma-glutamyl cysteine and glycine. Unfortunately, the existence of such a sequence has not been investigated in cardiac tissue, and the requirement for ATP implies that the process is too slow and limited to be of importance during the time course of the experiments described earlier. However, the possibility that a GSH uptake system does exist in the heart provides a wide and interesting scope for future studies. At present, the antiarrhythmic effects of exogenous GSH cannot be explained from the results described; an anti-free radical mode of action cannot be discounted. It would appear, though, that dilator-mediated coronary steal is not an important antiarrhythmic factor in the isolated rat heart model.

Interestingly, it has also been shown that the other sulphydryl agents and the vasodilator drugs, which were not antiarrhythmic, also attenuated the reperfusion-induced rise in $^{86}Rb^{+}$ efflux. This infers that $^{86}Rb^{+}$ efflux is not exclusively the cause or the consequence of arrhythmogenic mechanisms. However, such a conclusion would be premature in the absence of data showing the effects of sulphydryl and vasodilator agents on $^{86}Rb^{+}$ uptake and efflux in both the ischaemic and non-ischaemic regions. Even though the efflux of $^{86}Rb^{+}$ was reduced upon reperfusion of the ischaemic region, this does not necessarily indicate that tissue heterogeneity was similarly reduced. Whilst a direct relationship between the magnitude of the induced fall in perfusion pressure and the attenuation of $^{86}Rb^{+}$ loss has not been clearly demonstrated, it would seem logical to suggest that dilation-induced redirection of coronary flow from ischaemic vessels upon reperfusion contributed to the lowering of $^{86}Rb^{+}$ efflux from this area of tissue. However, previous results (chapter 4) demonstrated falls in perfusion pressure with desferrioxamine and oxypurinol without accompanying reductions of $^{86}Rb^{+}$ efflux and so preclude vasodilation as a major factor in this response. The reduction of $^{86}Rb^{+}$ efflux by vasodilator drugs (NITRO and HYD) was concentration-dependent however, and so complicates further the interpretation of the relationship between $^{86}Rb^{+}$ efflux and the dilator effects of GSH, PSH, DTT, desferrioxamine and oxypurinol. Any explanation must be speculative, and it would appear that proportionality between dilation and efflux is the normal response which was modified by additional actions of the above drugs. Evidence associating

intracellular or membrane-bound thiol groups with the regulation of potassium transport has however, been cited by many authors. Meury and colleagues (1980) demonstrated that the oxidation of critical thiol groups involved in potassium gating causes an increased outflow of potassium through *Escherichia coli* membranes. The transport mechanism was thought to be channel-regulated, since thiol oxidation with N-ethylmaleimide did not inactivate potassium pump systems. In pancreatic islet cells, $^{86}Rb^{+}$ efflux is reduced by agents that increase intracellular NAD(P)H concentrations and by GSH itself (Henquin, 1980). Modifications of the $NAD^{+}/NADH$ ratio in intact human erythrocytes similarly modulates the activity of calcium-dependent potassium transport (Alverez *et al*, 1986). Increases in cellular levels of reduced nicotinamide dinucleotides, as a direct result of elevated lactate concentrations, increase the affinity of calcium-activated potassium channels for calcium, leading to enhanced uptake of $^{86}Rb^{+}$. Although Alvarez's group did not monitor $^{86}Rb^{+}$ efflux, their evidence indicates that calcium-dependent potassium transport is modulated by redox reactions. Despite the need for more detailed investigations of cellular thiol-disulphide interactions, and of their role in ion transport systems, it is suggested that modification of the redox state of free thiols by GSH, PSH and DTT in the present study significantly contributed to the observed attenuation of $^{86}Rb^{+}$ efflux following reperfusion and that these effects did not appear to be concentration-dependent owing to the superimposed effects of dilatation.

NITRO is believed to act by elevating intracellular cyclic GMP (cGMP) levels via activation of guanylate cyclase (Rapoport *et al*, 1981). cGMP has reciprocal actions to those of cAMP and is, as such, an important regulator of intracellular calcium concentrations. It is therefore likely that NITRO-induced alterations of cellular calcium mobilisation will alter the movements of other cations via cation exchange. The mode of action of HYD is less well understood, but it is believed to exert a direct effect on smooth muscle. Jacobs (1984) demonstrated an HYD-dependent inhibition of actin-myosin interaction in an *in vitro* myofibril preparation. The effects of the drug on cation fluxes have not been described.

The initial objective of the work described in this chapter was to determine whether GSH is antiarrhythmic in the isolated heart model by virtue of its vasodilator action. The results suggest that this was not the case, although the precise mechanisms involved have not been determined. Future work should aim at identifying and dissociating the anti-free radical properties of this drug from other less specific, though no less important, effects on cellular free thiols and regulatory redox reactions.

Finally, these data highlight the need for more detailed analyses of both efflux and uptake kinetics of $^{86}Rb^{+}$ in both ischaemic and non-ischaemic tissues. Data aquired from such studies should allow the conclusive identification and magnitude of the washout component following reperfusion and of the transport systems involved in reperfusion-induced alterations to potassium fluxes.

CHAPTER 6

6.1 INTRODUCTION: XANTHINE OXIDASE AND FREE RADICAL REACTIONS IN THE HEART

Data presented so far demonstrate a similarity between the effects of the exogenous administration of xanthine oxidase to isolated hearts and those of reperfusion following regional myocardial ischaemia. $^{86}Rb^{+}$ efflux rose following both xanthine oxidase administration and reperfusion (chapter 3). The former was reduced by SOD, and Zakaria (1985) attenuated the reperfusion-induced elevation of $^{86}Rb^{+}$ efflux using a combination of the free radical scavengers SOD, catalase and mannitol. Reperfusion of isolated rat hearts was also shown to produce a ferricytochrome c-reducing species at a time when reperfusion arrhythmias were initiated (Woodward and Zakaria, 1985); both ferricytochrome c reduction and arrhythmias were reduced in the presence of SOD. Bernier *et al* (1986) have since confirmed the antiarrhythmic properties of anti-free radical interventions. These findings provide considerable (albeit circumstantial) evidence that oxygen free radicals are involved in the genesis of arrhythmias and the elevation of $^{86}Rb^{+}$ efflux following coronary reperfusion in isolated rat hearts.

The experiments described in section 6.2 were undertaken to confirm that the xanthine/xanthine oxidase free radical-generating system was indeed generating oxygen radicals in the hearts. It was necessary to establish this in order to give credence to the effects of the system on $^{86}Rb^{+}$ efflux (chapter 3) being ascribed to oxygen radicals. Xanthine oxidase has itself been postulated as an

endogenous source of free radicals during ischaemia/reperfusion. Roy and McCord (1983) showed that the conversion of xanthine dehydrogenase (which utilises NAD^+ as an electron acceptor) to the oxidase (which utilises O_2 as an electron acceptor) occurs in many tissues. 50% conversion to the oxidase in the rat heart was seen within 5 min of ischaemia, a time corresponding to the peak duration of ischaemia required to evoke the highest incidence of reperfusion arrhythmias in this species (Manning and Hearse, 1984). Roy and McCord (1983) proposed that the substrate-starved and oxygen-deprived tissue is depleted of high energy phosphates such as ATP. ATP is catabolised to AMP, adenosine, inosine and finally hypoxanthine and xanthine, the substrates for xanthine oxidase. The fall in energy levels in the cell is proposed to result in a redistribution of cations which Roy and McCord suggested causes an increased calcium influx that activates intracellular proteases capable of converting the dehydrogenase to the oxidase. Soybean trypsin inhibitor (STI), which inhibits the activity of proteases, has indeed been shown to prevent enzymic conversion in rat intestine (Roy and McCord, 1983). The cell, having accumulated the substrates (hypoxanthine and xanthine) and the enzyme (xanthine oxidase), is now "primed" for the production of free radicals when the final substrate, O_2, becomes available (i.e. upon reperfusion).

The above scheme of events is attractive and it is tempting to extrapolate its occurrence to the heart. In fact allopurinol and folic acid, both inhibitors of xanthine oxidase, were shown to reduce the incidence of reperfusion arrhythmias in the rat (DeWall *et al*, 1971; Manning *et al*, 1984). However, the distribution of

xanthine oxidase varies in different tissues of the same species and in the same tissues in different species (Parks and Granger, 1986). Whilst the enzyme may be important in the genesis of reperfusion arrhythmias in the rat, its activity is negligable in the rabbit heart (Schoutsen and de Jong, 1987) and is yet to be demonstrated in the human heart. The final study to be described was therefore aimed at establishing the presence, location and activity of xanthine dehydrogenase and xanthine oxidase in cardiovascular cells. Where possible, cells derived from rat hearts were used, but since rat coronary smooth muscle and endothelial cells were not easily available, rat aortic smooth muscle cells and human umbilical endothelial cells were substituted. Enzyme detection involved the measurement of xanthine oxidase-derived oxygen radicals using luminol-enhanced chemiluminescence. This technique has been used by others (Miura and Ogiso, 1985) to measure xanthine oxidase activity in cell-free systems.

6.2 RESULTS: THE XANTHINE/XANTHINE OXIDASE SYSTEM

6.2.1 Demonstration of Free Radical Generation by Xanthine Oxidase, and Free Radical Scavenging by SOD, in Isolated Non-ligated Rat Hearts

Table 6.2.1(a) shows the percentage increase in the rate of ferricytochrome c (FeCyt c) reduction (i.e. % optical density increase per min; %dE/min) induced by xanthine oxidase in non-ligated hearts. In the absence of the enzyme's substrate xanthine, bolus additions of xanthine oxidase (0.65 U and 1.30 U) caused dE/min to increase by 14±2% and 17±3% respectively. During perfusion with 0.1mM xanthine, 0.65 U xanthine oxidase elevated dE/min by 44±2%, a significant rise when compared with the 1±1% increase observed with the enzyme vehicle (3.2M ammonium sulphate/0.02% sodium salicylate). The change achieved with 0.65 U was apparently maximal, since 1.30 U did not produce a significantly greater change.

Concomitant perfusion with SOD (10 U/ml) significantly attenuated resposes to xanthine oxidase in the presence and absence of xanthine (table 6.2.1(a)).

There appeared to be sufficient substrate in hearts to allow some radical generation, since FeCyt c was reduced by xanthine oxidase in the absence of exogenous xanthine. This is demonstrated further by the results presented in table 6.2.1(b), which were obtained from identical perfusion experiments in the absence of hearts. With

PERFUSATE COMPOSITION		BOLUS (0.01ml) ADDITIONS			MEAN % INCREASE OF E550nm ± sem	
XANTHINE (0.1mM)	SOD (10 U/ml)	VEHICLE	XANTHINE OXIDASE 0.65 U	1.30 U	(a)	(b)
-	-	+	-	-	0±0	0±0
-	-	-	+	-	14±2*	1±1[b]
-	-	-	-	+	17±3*	0±0[b]
+	-	+	-	-	1±1	0±0
+	-	-	+	-	44±2*	48±2*
+	-	-	-	+	45±3*	52±2*
-	+	+	-	-	0±0	0±0
-	+	-	+	-	3±1[a]	1±1
-	+	-	-	+	7±1*[a]	1±1[b]
+	+	+	-	-	2±1	0±0
+	+	-	+	-	15±3*[a]	21±1*[a]
+	+	-	-	+	20±2*[a]	28±2*[a]

Table 6.2.1:

The reduction of ferricytochrome c (mean percentage increase of E550nm) in (a) coronary effluent from isolated rat hearts and (b) perfusion fluid which has not passed through hearts (n=3 per group).

+/- represents the presence/absence of each agent.

* $p<0.05$ compared with the vehicle control value
a $p<0.05$ compared with the corresponding value in the absence of SOD
b $p<0.05$ compared with the corresponding value in column (a).

this protocol, xanthine oxidase (0.65 U and 1.30 U) had a negligable effect on the rate of optical density change (1±1% and 0±0% respectively) when xanthine was omitted from the perfusate. These changes were significantly smaller than those observed in experiments using hearts (14±2% and 17±3% respectively; table 6.2.1(a)). The inhibitory effect of SOD was unaffected in these experiments.

Notabley higher rates of optical density change were obtained with xanthine/xanthine oxidase when hearts were not used in these experiments (table 6.2.1). It is possible that the heart tissue in the previous experiments provided a site for radical chain termination reactions, such as lipid peroxidation, which would have competed for the reaction site on the FeCyt c molecule, thus lowering the observed rate of FeCyt c reduction.

These results demonstrate that the xanthine/xanthine oxidase system generates species in isolated rat hearts that are scavenged by SOD. Since SOD is specific for the dismutation of $O_2 \cdot^-$ (Halliwell and Gutteridge, 1985), this radical was likely to be the species in question.

6.2.2 Purification of Commercial Xanthine Oxidase

In the above experiments, commercially obtained xanthine oxidase was shown, in the presence of xanthine, to generate free radicals, and was previously shown to elevate $^{86}Rb^+$ efflux in non-ligated hearts (chapter 3, section 3.2.4). The vehicle in which the enzyme

is supplied comprises 3.2M ammonium sulphate and 0.02% sodium salicylate. Freshly prepared solutions of vehicle did not affect FeCyt c reduction (table 6.2.1) or $^{86}Rb^{+}$ efflux, as demonstrated by the results described in section 3.2.4 and illustrated in bar chart form in figure 6.2.1. However, Fridovich (1982) has reported that exposure of xanthine oxidase to very high salt concentrations may deflavinate the enzyme to a form (deflavoxanthine oxidase) that directly reduces FeCyt c. It was necessary to demonstrate that responses to xanthine oxidase were $O_2\cdot^-$-mediated. The enzyme vehicle was therefore removed by dialysis in 0.9% (w:v) saline, or by gel filtration using a Sephadex G-25 column.

Following gel filtration of xanthine oxidase, eluent samples were assayed for their relative protein content and subjected to a barium sulphate precipitation test to identify samples that contained vehicle sulphate (figure 6.2.2). A good separation was achieved between protein-containing samples and samples that were barium sulphate positive. Samples were pooled into four fractions (figure 6.2.2), assayed for protein and, together with dialysed enzyme samples, subjected to the standard cell-free FeCyt c assay for determination of xanthine oxidase activity. Table 6.2.2 shows that the activity of the desalted enzyme (fraction 1) compared favourably with the activity of the commercial enzyme sample (0.50 ± 0.02 and 0.054 ± 0.01 absorbance units/min/mg protein respectively). No enzyme activity was observed in fractions 2 to 4. Dialysed enzyme samples exhibited enzyme activities which were of the same order of magnitude as those seen with the commercial enzyme samples (table 6.2.2). These data demonstrate that the

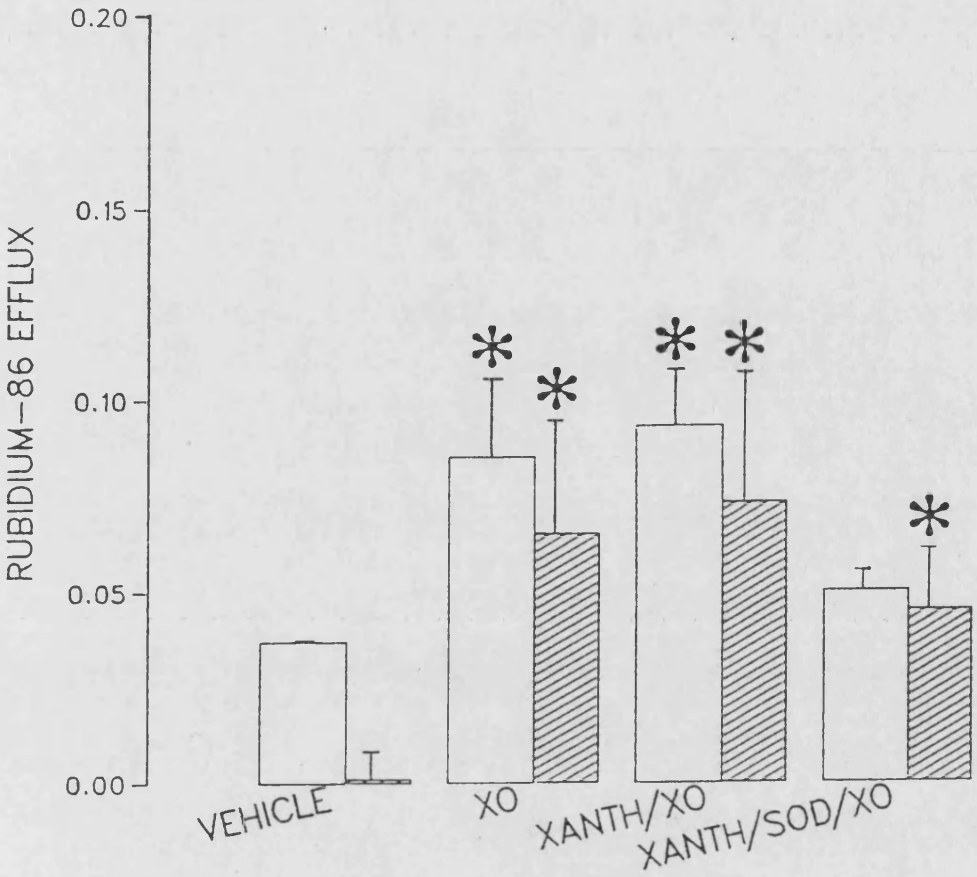


Figure 6.2.1:

$^{86}Rb^{+}$ efflux in non-ligated hearts induced by bolus additions (0.01ml) of 0.65U xanthine oxidase, alone (XO, n=4), in the presence of 0.1mM xanthine (XANTH/XO, n=5) or in the presence of 0.1mM xanthine and 10U/ml SOD (XANTH/SOD/XO, n=6). The control group (VEHICLE) was administered 0.01ml of enzyme vehicle (n=3).

Mean and standard errors are shown for peak erc (open bars) and efflux area values (shaded bars).

* $p<0.05$ compared with the control group.

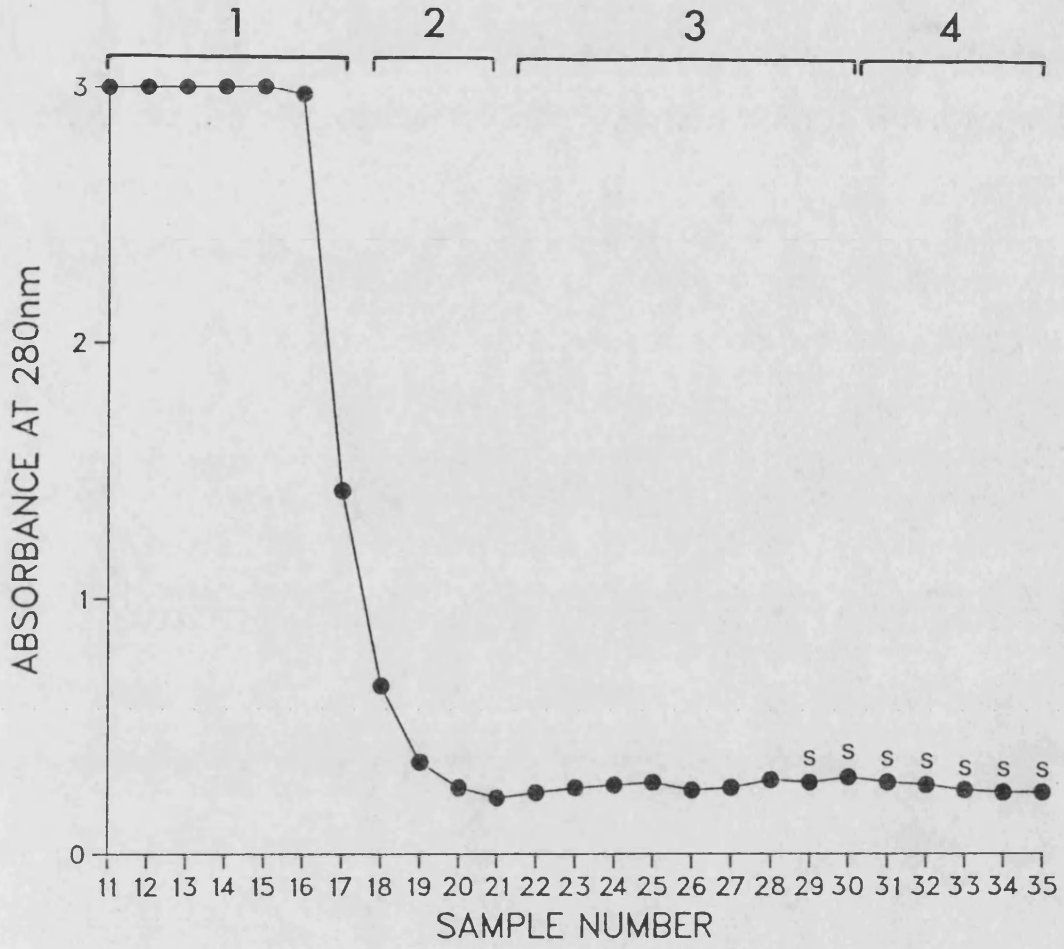


Figure 6.2.2

Relative protein content (E280 nm) of eluent samples of commercially available xanthine oxidase following Sephadex G-25 gel filtration (n = 1).

Each tube contained 1 ml of eluent collected over 2 min.

Samples shown to contain sulphate are denoted by S.

SAMPLE (0.01ml)	RATE FeCyt c REDUCTION (abs. unit/min/mg protein) ±sem
COMMERCIAL XO (0.65 U)	0.54±0.01
FRACTION 1	0.50±0.02
FRACTION 2	0 ±0
FRACTION 3	0 ±0
FRACTION 4	0 ±0
DIALYSED XO	0.45±0.01

Table 6.2.2:

The reduction of ferricytochrome c by xanthine oxidase (XO). Samples of commercially available enzyme were tested before and after (fractions 1-4) Sephadex G-25 gel filtration or dialysis.

Values are the rate of optical density change (at 550nm) per milligramme of total sample protein. The mean and standard error of 2 spectrophotometric determinations are shown for each assay group.

vehicle for commercial xanthine oxidase did not contribute to the reduction of FeCyt c, indicating that the commercial preparation reduced FeCyt c via a $O_2\cdot^-$-dependent mechanism. The activities of desalted and commercial samples of the enzyme did not significantly differ.

6.3 RESULTS: THE LOCATION AND ACTIVITY OF XANTHINE OXIDASE AND XANTHINE DEHYDROGENASE IN CARDIOVASCULAR CELLS

The *in situ* generation of free radicals by xanthine oxidase in the heart may contribute to the genesis of reperfusion arrhythmias (section 6.1). This hypothesis assumes the existence of the enzyme in hearts. A study was therefore undertaken, using cultured cardiovascular cells, to determine the locations and activities of xanthine oxidase and dehydrogenase. Luminol-enhanced chemiluminescence detection of free radicals generated by xanthine oxidase was carried out on rat aortic vascular smooth muscle (vsm) cells, adult rat cardiac myocytes, neonatal rat cardiac myocytes, rat cardiac fibroblasts and human umbilical vein endothelial cells. Xanthine dehydrogenase (which does not generate free radicals) was detected after sulphydryl oxidation to the oxidase with DTNB (Clare *et al*, 1981). Both enzyme activities were measured within 4 min of lysing the cells.

6.3.1 Verification of the Generation and Detection of Superoxide or Superoxide-derived Free Radicals

The addition of increasing quantities of xanthine oxidase to cell

medium produced dose-dependent increases in instantaneous chemiluminescence, as has been observed in cell-free systems (Miura and Ogiso, 1985). This relationship was linear up to 10 mU xanthine oxidase (figure 6.3.1). The inclusion of 10 U SOD in these experiments significantly reduced photon emission at all time points (figure 6.3.2), indicating that increased photon emission following xanthine oxidase addition was the result of $O_2\cdot^-$ or $O_2\cdot^-$-derived radical formation.

The addition of 5 mU xanthine oxidase to cell media (DMEM, HAMS F10 (x10) and medium 199) containing 0.4mM xanthine produced increases in photon emission (31±1 cps, 33±1 cps and 33±1 cps respectively) which did not significantly differ, indicating that the 3 media had similar antioxidant properties.

6.3.2 Chemiluminescence Emission from Cells

Photon emission from each of the 5 cell preparations is shown in figure 6.3.3(A to E). In none of the cells was xanthine oxidase activity detected on the extracellular surface, since chemiluminescence did not increase on addition of luminol.

Following cell lysis with Triton-X100, chemiluminescence was detected primarily in endothelial cells (figure 6.3.3D), although smaller but significant increases in count rate were also observed in the other cell types. Chemiluminescence on addition of DTNB occurred mainly in endothelial cells, with the other cells exhibiting much smaller responses. At the end of each experiment,

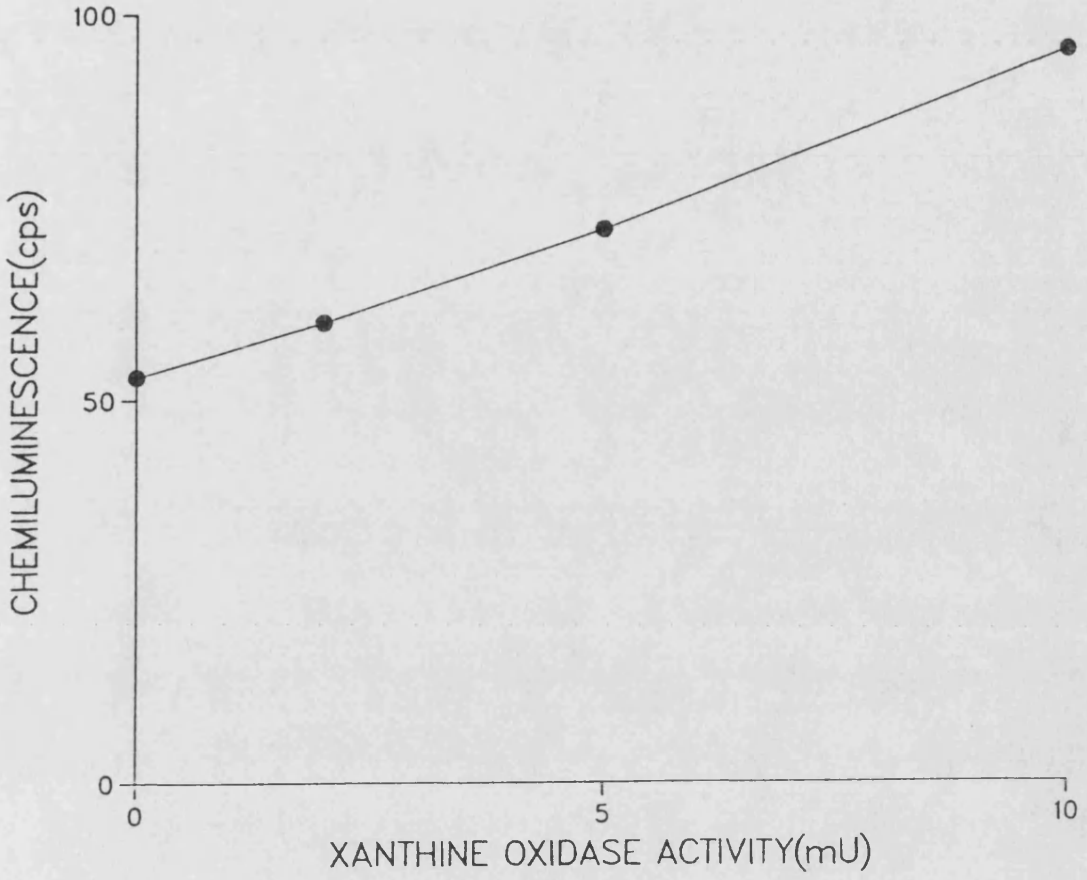


Figure 6.3.1

The increase in chemiluminescence (counts per second, cps) induced by increasing quantities of xanthine oxidase.

Each point is the mean change of 10 counts. Standard error bars are smaller than each data point.

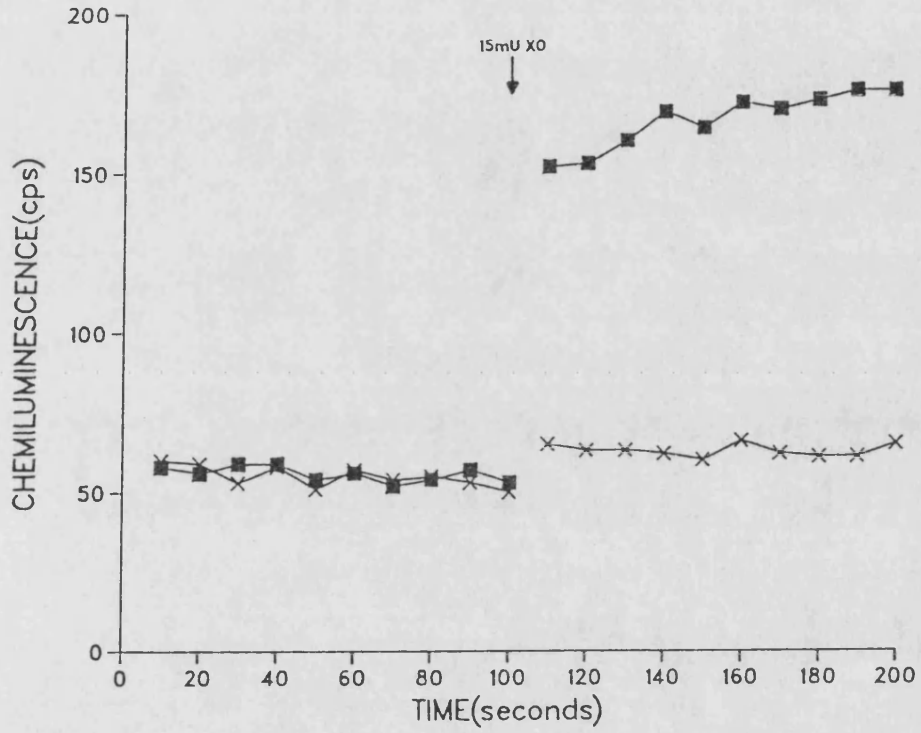


Figure 6.3.2

Chemiluminescence induced by the addition of 15 mU xanthine oxidase in DMEM medium containing 0.4 mM xanthine and 2 mg/ml luminol.

Experiments were carried out in the presence (X) and absence (■) of 10 U SOD (n = 1 per group).

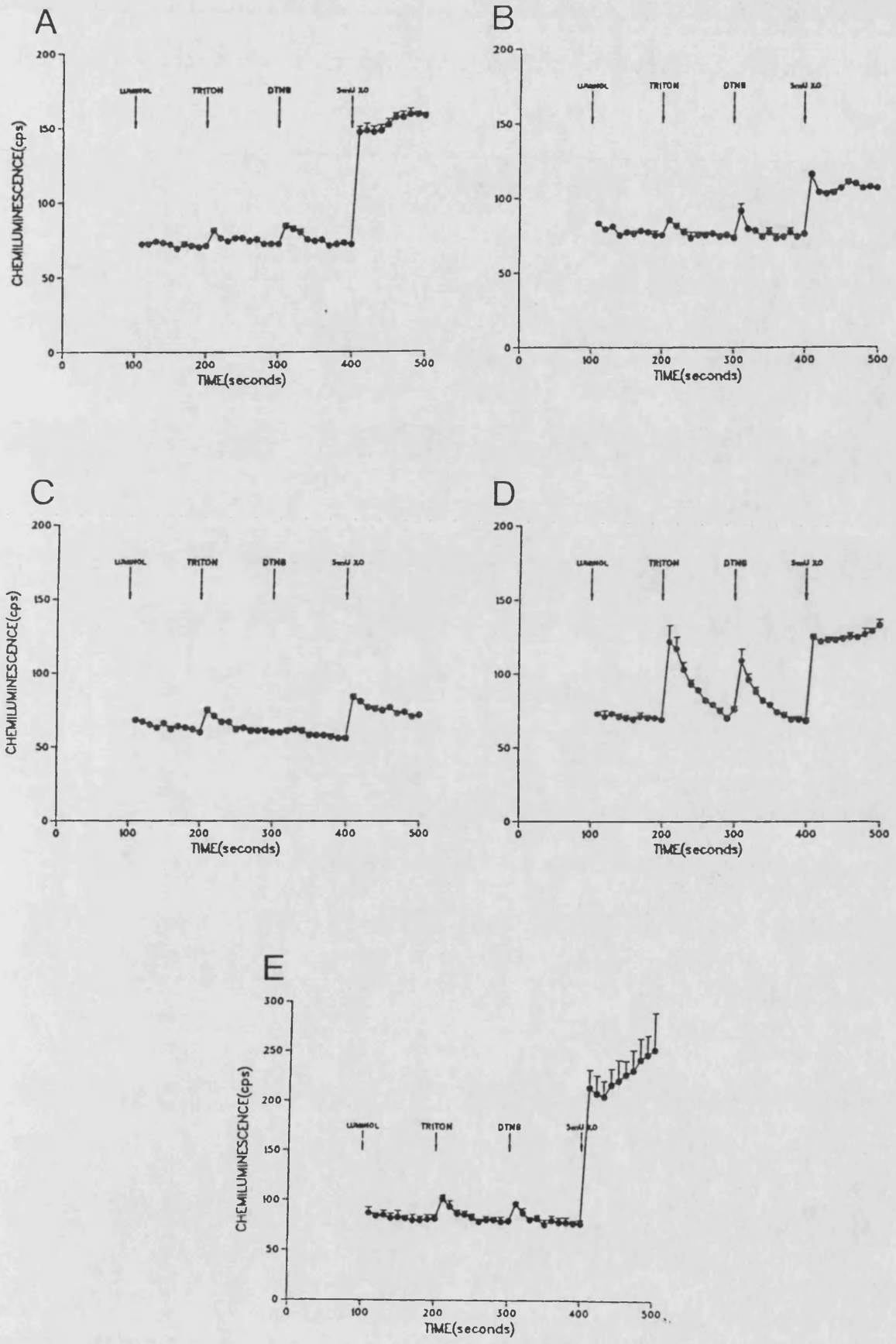
Figure 6.3.3:

Chemiluminescence emission from 5 cell preparations.

Measurements were made in the presence of 0.4mM xanthine on (A) adult rat myocytes, (B) neonatal rat myocytes, (C) rat aortic (A10) vsm cells, (D) human umbilical vein endothelial cells, and (E) rat cardiac fibroblasts.

Additions of 0.04ml volumes of luminol (2mg/ml), triton-X100 (1% v:v), 5,5'dithiobis-2-nitrobenzoic acid (DTNB, 5mM), and xanthine oxidase (XO, 5mU) were made at the times indicated.

The mean and standard error of triplicate measurements are shown.


A
B
C
D
E
TRITON
DTNB
CHEMILUMINESCENCE(cps)
TIME(seconds)

5 mU xanthine oxidase were added as a positive control to correct for different quenching of chemiluminescence by the media and phototube responsiveness on the day of the experiment. It was also used, in conjunction with protein determinations on different but similarly prepared plates of cells, to quantify the activities of xanthine oxidase and xanthine dehydrogenase.

6.3.3 Xanthine Oxidase/Dehydrogenase Activities in Cultured Cells

Substantial xanthine oxidase activity was observed in endothelial cells (27.4±7.2 mU/mg protein; table 6.3.1). Neonatal myocytes and vsm cells exhibited less activity, whilst adult myocytes and cardiac fibroblasts showed negligable enzyme activity.

Xanthine dehydrogenase-derived oxidase activity was again highest in endothelial cells (16.5±5.8 mU/mg protein), and the ratio of dehydrogenase to oxidase activity in these cells was 0.6:1. The ratio in vsm cells was approximately 0.3:1. Neonatal myocytes were peculiar in exhibiting a greater dehydrogenase to oxidase activity (2:1). Adult myocytes and cardiac fibroblasts had very low enzyme activities in a 1:1 ratio (table 6.3.1).

In order to confirm that the substantial chemiluminescence observed in the human endothelial cells arose from xanthine oxidase activity, experiments on these cells were repeated in the presence and absence of 1μM oxypurinol (figure 6.3.4). The xanthine oxidase inhibitor significantly reduced photon emission at all time points after additions of Triton-X100 and DTNB.

CELL TYPE	ENZYME ACTIVITY (mU/mg total cell protein)	
	XANTHINE OXIDASE	XANTHINE DEHYDROGENASE
HUMAN ENDOTHELIAL CELLS	27.4±7.2	16.5±5.8
ADULT MYOCYTES	0.4±0.3	0.5±0.2
A10 VSM CELLS	15.4±2.8	4.4±2.4
CARDIAC FIBROBLASTS	2.2±0.5	1.8±0.2
NEONATAL MYOCYTES	4.7±0.3	8.0±4.5

Table 6.3.1:

Xanthine oxidase and xanthine dehydrogenase activities in 5 cardiovascular cell preparations.

Values are the mean and standard error of 3 experiments.

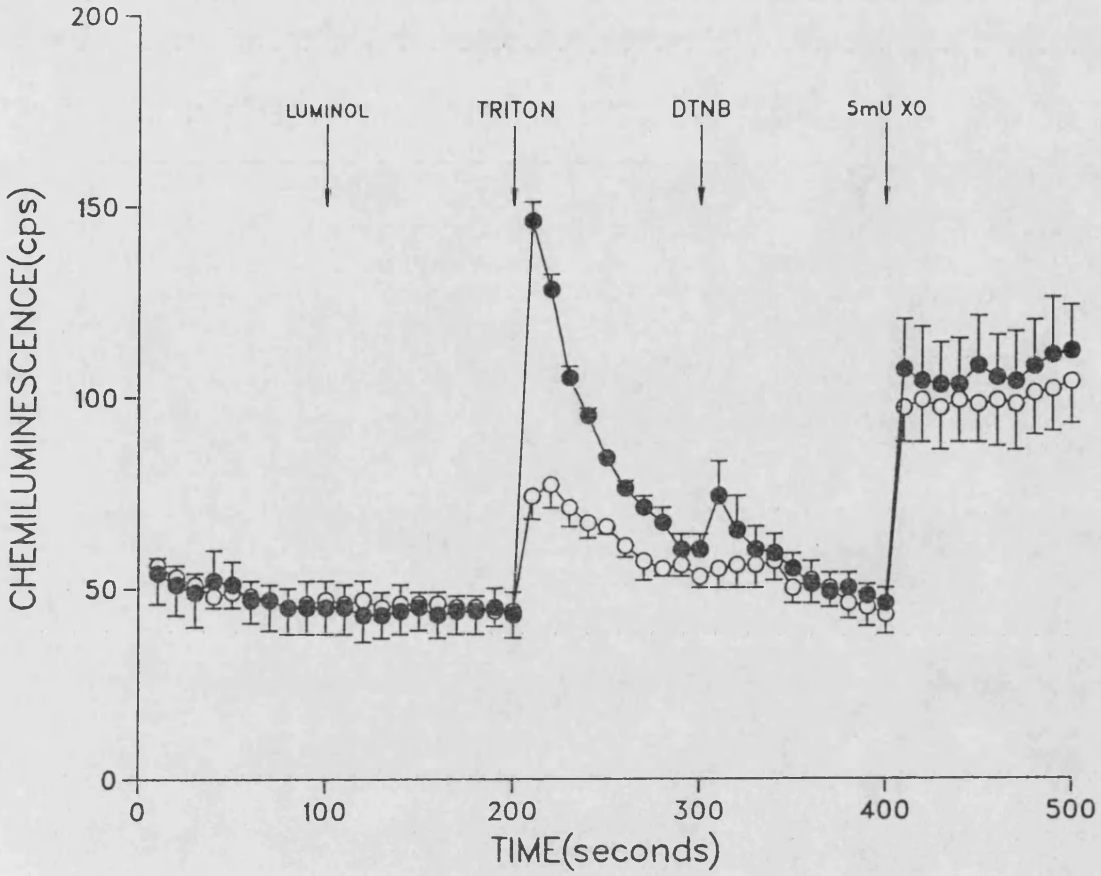


Figure 6.3.4:

Chemiluminescence emission from human endothelial cells in the presence (O) and absence (●) of 1μM oxypurinol.

Cells were bathed in medium 199 containing 0.4mM xanthine. Additions of 0.04ml volumes of luminol (2mg/ml), triton-X100 (1% v:v), 5,5'dithiobis-2-nitrobenzoic acid (DTNB, 5mM) and xanthine oxidase (XO, 5mU) were made at the times indicated.

The mean and standard error of triplicate experiments are shown.

6.4 DISCUSSION

The results presented demonstrate that the xanthine/xanthine oxidase free radical generating system, first characterised by McCord and Fridovich (1968), did in fact produce $O_2 \cdot^-$ or $O_2 \cdot^-$-derived radicals in isolated hearts. The vehicle in which the commercially prepared enzyme is presented did not affect FeCyt c reduction and so it can be stated with some degree of certainty that the responses of isolated hearts following administration of the enzyme (chapter 3) were the direct result of oxygen radical generation. This conclusion is supported further by the work of Zakaria (1985), who abolished the xanthine oxidase-induced reduction of FeCyt c by previously inactivating (boiling) the enzyme. In the absence of more direct methods for measuring $O_2 \cdot^-$ production, the FeCyt c assay has proved to be a good alternative for qualitative and quantitative radical measurement in perfused tissue systems.

It has also been shown that luminol-enhanced chemiluminescence is both a rapid and reproducible technique for the assay of xanthine oxidase and xanthine dehydrogenase in small quantities of cells. Measurements of the instantaneous chemiluminescence derived from the cells were successfully used to quantify enzyme activities even though the signals were transient. It is possible that the instantaneous value is artificially high because of locally high concentrations of enzyme around the newly lysed cells. However, this is most unlikely since a transient signal was also observed

when DTNB was added to the, by then, homogeneous lysate suspension. Whilst transient increases in chemiluminescence have also been observed in solution measurements of xanthine oxidase activity (Miura and Ogiso, 1985), the possibility remains that the transient increases in photon emission obtained following cell lysis or on addition of DTNB were the result of varying inherent radical scavenging capacities of the cells. Addition of 5 mU xanthine oxidase, the positive control, produced photon emission which (with the exception of vsm cells) tended to rise over the interval observed. This may indicate that the endogenous antioxidant properties of the cells were overwhelmed at this concentration of enzyme. Figure 6.3.1 provides some indication that the rate of increase of chemiluminescence is proportional to the activity of the added enzyme in the absence of cells.

The data that show the majority of xanthine oxidase activity to be located in endothelial and vsm cells is in agreement with previous qualitative experiments (Jarasch *et al*, 1981; Bruder *et al*, 1982; Werns *et al*, 1986). Xanthine dehydrogenase is similarly located overwhelmingly in the vasculature, with the exception of neonatal myocytes. These findings imply that the greatest generation of free radicals by endogenous xanthine oxidase would occur in the vasculature, even if xanthine dehydrogenase is a potential "store" of oxidase for radical production under pathological conditions. It is interesting to note that neonatal myocytes are peculiar in exhibiting a high dehydrogenase:oxidase ratio. This may represent a developmental stage leading to the reverse situation in the adult myocyte in rats. Recently, Schoutsen and de Jong (1987)

demonstrated an age-dependent increase in xanthine oxidoreductase (xanthine oxidase plus xanthine dehydrogenase) activity in cultured rat myocytes. The authors did not find any variation in the oxidase:dehydrogenase ratio with age. Whilst these findings conflict with those presented here, it should be noted that the assay system used by Schoutsen and de Jong was both rigorous and time-consuming, and may not reflect the true enzyme activities of the cells studied. However, it must be said that the high dehydrogenase:oxidase ratio observed in neonatal myocytes in the present study was not common to all of the neonatal cells used. This was true for both cardiac fibroblasts (derived from the same animals as the neonatal myocytes) and endothelial cells (derived from human umbilical vein) although the latter, of course, is of a different species origin.

It could be argued that the vascular location of xanthine oxidase-derived free radicals, indicated by the present study, leads to the formation of ion gradients. The studies described in chapter 3 demonstrated increased $^{86}Rb^{+}$ (potassium) efflux upon reperfusion of isolated rat hearts which were reproduced by the administration of xanthine oxidase both in the presence and absence of xanthine. It is possible that endogenous xanthine oxidase induces $^{86}Rb^{+}$ efflux via free radical generation and that the resultant ionic heterogeneity is arrhythmogenic. Two arguments tend to refute this proposal however. Firstly, the xanthine oxidase inhibitor oxypurinol, which was effective at inhibiting the enzyme in the cell experiments, was without effect on $^{86}Rb^{+}$ efflux in isolated heart preparations. Secondly, Schoutsen and de Jong (1987)

have shown that rabbit hearts exhibit nominal xanthine oxidase activity but are still vulnerable to reperfusion arrhythmias. Therefore, in the absence of further supportive data, it is impossible to conclude a role for xanthine oxidase in mechanisms of reperfusion arrhythmogenesis. An investigation of the action of xanthine oxidase-derived free radicals on $^{86}Rb^{+}$ fluxes in cardiovascular cells from various animal species including the rabbit may help to resolve this anomoly.

The presence of xanthine oxidase in human cardiovascular cells has yet to be demonstrated. The applicability of data arrived at using animal cells (in this case, predominantly from the rat heart) to the human situation cannot therefore be assumed. There is an obvious requirement for the application of the methods used here to human cells derived from adult donors. Such studies would clarify further the role of xanthine oxidase and xanthine dehydrogenase in the mechanisms of reperfusion arrhythmogenesis.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The results presented in chapter 3 confirmed the observations of others (Woodward and Zakaria, 1985; Bernier *et al*, 1986) that reperfusion of regionally ischaemic isolated rat hearts evokes life-threatening ventricular arrhythmias. The above authors also showed known anti-free radical agents to be antiarrhythmic, whereas in the present study, the only anti-free radical drug that exhibited similar properties was glutathione (chapter 4). The link between free radicals and arrhythmias relies mainly upon circumstantial evidence, a reflection of the difficulties involved in measuring short-lived chemical species such as $O_2 \cdot^-$ and $OH \cdot$. An understanding of the modes of action of radical scavengers with their antiarrhythmic effects is still lacking and the results of this study highlight the need to carefully examine other seemingly untoward actions of the compounds studied. For example, glutathione is a powerful coronary dilator (chapters 4 and 5) and its antiarrhythmic actions may therefore be more closely related to a coronary steal mechanism than to protection against free radicals. However, by comparing the antiarrhythmic and dilator properties of glutathione with those of other sulphydryl compounds (d-penicillamine and dithiothreitol) and vasodilator drugs (sodium nitroprusside and hydralazine), it becomes apparent that dilation is not the predominant antiarrhythmic mechanism of glutathione (chapter 5 and table 7.1). Similarly, the effects of desferrioxamine and hypoxia on the incidence of reperfusion arrhythmias were negligible despite accompanying dilator responses (chapter 4 and table 7.1), and while these results tend to negate the importance of oxygen radicals to reperfusion arrhythmias, they serve to highlight further the non-dilator-mediated antiarrhythmic

action of glutathione.

Antiarrhythmic drugs must ultimately preserve the ionic homeostasis of the heart. By measuring the efflux of potassium using $^{86}Rb^{+}$, reperfusion-induced arrhythmias were shown to be accompanied by a substantial loss of this ion. Initially, this effect was thought to be of potential importance to the mechanisms of the observed arrhythmias, since it appeared not to involve the passive washout of accumulated ions from the ischaemic region (chapter 3). However, table 7.1 clearly shows that a reduction of $^{86}Rb^{+}$ efflux is not always associated with protection against arrhythmias but is more closely related to the magnitude of the dilation induced by the drug in question. Even so, this relationship is not exclusive to all compounds studied, the exceptions being desferrioxamine and oxypurinol. Indeed, the concentration-dependent dilator action of glutathione was not accompanied by a concentration-dependent reduction of $^{86}Rb^{+}$ efflux; there appeared to be a threshold of dilation above which the outward movement of $^{86}Rb^{+}$ was reduced. Whilst speculative explanations for these effects can be presented (the possible role of thiol-disulphide interactions, for example; see chapter 5), no definitive conclusions can yet be drawn. Changes in the efflux of an ion in any given area of tissue do not necessarily reflect the uptake kinetics of that ion. Until both are known, ionic heterogeneity between areas of tissue cannot be assumed. Similarly, the relative contributions of vascular and cardiac tissue to $^{86}Rb^{+}$ efflux are unknown. A continuation of this study must resolve these ambiguities since, until this is done, it is impossible to conclude the existence (or non-existence) of a

relationship between arrhythmogenesis and $^{86}Rb^{+}$ efflux from the present study.

The origin of reperfusion-induced arrhythmias is almost certainly multi-factorial. With accumulating evidence of the production of free radicals upon reperfusion, it is reasonable to suggest that these reactive species, with their potential to disrupt membrane integrity, are one such factor. Whether oxygen free radicals are the cause and/or initiators of arrhythmias remains to be seen. The data presented in chapter 4 suggests they are not of prime importance, since by removing the main substrate (oxygen) and catalysts (Fe^{2+} and Fe^{3+}) for their production by inducing hypoxia or perfusing with desferrioxamine respectively, the incidence of arrhythmias remained at or near the control values. However, the experimental generation of oxygen radicals (with xanthine/xanthine oxidase) increased $^{86}Rb^{+}$ efflux in non-ligated hearts by a similar magnitude to that observed following reperfusion. The absence of arrhythmias in these experiments points to a dissociation between $^{86}Rb^{+}$ loss and arrhythmogenesis, although an heterogeneic rather than global generation of radicals (and hence elevation of $^{86}Rb^{+}$ efflux) may be a prerequisite for the development of arrhythmias (chapter 3).

The allopurinol study (chapter 4) reaffirmed the need to assess the precise antiarrhythmic mechanisms of anti-free radical drugs. In the isolated rat heart, the xanthine oxidase inhibitor allopurinol exhibited substantial antiarrhythmic properties. The active metabolite of allopurinol, oxypurinol, was without effect on the

incidence of arrhythmias, indicating that allopurinol protects against rhythm disturbances by mechanisms which are unrelated to inhibition of xanthine oxidase. Recently (Das *et al*, 1987), a direct radical scavenging mechanism has been postulated for allopurinol and oxypurinol, both of which demonstrated antiarrhythmic properties. A role for endogenous xanthine oxidase as a source of arrhythmogenic free radicals is also unlikely, owing to the fact that animal species which possess very little enzyme (e.g. the rabbit) are still vulnerable to reperfusion arrhythmias. In the rat, however, the vascular location of xanthine oxidase (chapter 6) may be of importance in the pathogenesis of microvascular disorders which would tend to exacerbate the effects of ischaemia.

One question that appears to be of crucial importance to this whole area of study remains to be answered adequately. Do free radicals or radical generating systems induce changes in action potential characteristics of arrhythmogenic proportions? Surprisingly, only one recent study (Barrington *et al*, 1985) has attempted to answer this. The results demonstrated that an iron-based radical generating system evoked severe electrophysiological abnormalities upon reoxygenation of cultured myocytes. Future studies, using multiple electrode techniques in whole hearts under conditions of ischaemia/reperfusion, should help to elucidate the temporal and spatial electrophysiological responses to free radicals in the way that the effects of ischaemia were investigated by Pogwizd and Corr (1987).

The problems raised by the current study, together with suggestions for extension of the work, are cited within the appropriate chapter discussions. The tactic of screening anti-free radical agents for antiarrhythmic properties has provided circumstantial evidence for and against an involvement of oxygen radicals in reperfusion-induced arrhythmogenesis. Now may be a prudent time to begin to characterise, in detail, each individual drug, to examine its effect on cardiac electrophysiology and function and to establish the relevance of its anti-free radical role in its protection against reperfusion-induced arrhythmias.

DRUG		ANTIARRHYTHMIC PROPERTIES	REDUCTION OF $^{86}Rb^{+}$ EFFLUX	DILATOR RESPONSE
GLUTATHIONE	10^{-5}M	*	0	**
	10^{-4}M	*****	0	****
	10^{-3}M	***	*****	*****
PSH	10^{-4}M	0	*****	*****
	10^{-3}M	0	*****	****
DTT	10^{-3}M	0	*	***
NITRO	10^{-8}M	0	****	***
	10^{-7}M	0	****	****
HYDRALAZINE	10^{-4}M	****	*****	*****
DESF	10^{-4}M	0	0	****
	10^{-3}M	0	0	**
OXYPURINOL	10^{-6}M	0	0	***
6-OHDA	60mg/kg	*	****	-
ANOXCAL		0	0	*****
ANOXREP		0	>control	***
ALLOPURINOL		****	0	-

Table 7.1:

Summary of the effects of drugs used in the study in terms of their antiarrhythmic properties, their ability to reduce the reperfusion-induced elevation of $^{86}Rb^{+}$ efflux and their dilator actions.

Antiarrhythmic properties were assessed as the extent by which the incidences of VT and VF were reduced, and the degree by which the onset was delayed and the duration reduced for each type of arrhythmia.

Symbols denote the speculative scores for each parameter;

0	no effect
* to *****	increasing effectiveness of drug
-	not assessed

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APPENDIX

PROGRAMME FOR CALCULATION OF $^{86}Rb^{+}$ EFFLUX RATE COEFFICIENTS

The following computer programme was used to calculate $^{86}Rb^{+}$ efflux rate coefficients (erc's) with data obtained from liquid scintillation counting of perfusate and tissue samples. The programme was modified from that of Dr.M.Coldwell (Beechams Pharmaceuticals plc., Harlow, Essex) and was written for use on the BBC Master Series microcomputer.

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ryanodine

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 10
 20  REM   INDIVIDUAL RUBIDIUM EFFLUX PROGRAMME
 30  REM
 40  @%=10
 50  CLEAR
 60  CLS
 70  VDU2
 80  PRINT TAB(12)"RUBIDIUM EFFLUX"
 90  PRINT TAB(12)STRING$(15,"=")
100  VDU3
110  INPUT"DATE (DD-MM-YY) "DATE$
120  VDU2
130  PRINT"DATE  "DATE$
140  VDU3
150  REM        DATA ENTRY
160  REM     AND CALCULATIONS
170
180  INPUT "CONDITIONS " CONDITIONS$
190  VDU2
200  PRINT"CONDITIONS " CONDITIONS$
210  VDU3
220  INPUT "NUMBER OF EFFLUX TUBES" E
230  DIM A(E),B(E),D(E),L(E),M(E),P(E),R(E)
240  INPUT"START TIME(MINS)" T
250  *FX229,1
260  PRINT"ENTER EFFLUX COUNTS"
265  PRINT"AND COLLECTION PERIOD....."
270  FOR N=1 TO E
280    PRINT N;
290    INPUT A(N);L(N)
300    R(N)=R(N-1)+L(N)
310  NEXT
320  REM   INCORRECT VALUE TRAP
330
340  CLS
350  INPUT "ARE THE VALUES CORRECT?(Y/N) " YES$
360  IF YES$= "Y" THEN 490
370  CLS
380  INPUT "WHICH SAMPLE NUMBER IS WRONG? "WRONGSAMP
390  INPUT "WHAT IS THE CORRECT VALUE?  " RIGHTVAL
400  A(WRONGSAMP)=RIGHTVAL
410  CLS
420  FOR N=1 TO E
430    PRINT N,A(N)
440    IF N=20 REPEAT:PRINT"PRESS SPACE BAR TO CONTINUE":UNTIL GET=32:CLS
450  NEXT N
460  INPUT "NOW ARE THE VALUES CORRECT? (Y/N) "YES2$
```

```
470  IF YES2$="Y" THEN 490
480  GOTO 370
490  CLS
500  FOR N=1 TO E
510    B(N)=B(N-1)+(A(N)*L(N))
520  NEXT
530  PRINT'
540  INPUT "TISSUE COUNTS " C
550  PRINT
560  INPUT "TISSUE WEIGHT (mg)   " W
570  PRINT
580
590  *FX229,0
600  @%=&20309
610  FOR N=1 TO E
620    D(N)=(B(E)+C)-B(N-1)
630    P(N)=A(N)/D(N)
640  NEXT
650
660  REM        PRINTING
670  REM       OF RESULTS
680
690  VDU2
700  PRINT
710  PRINT"     EFFLUX ";"     CUMUL. ";"     TOTAL   ";"   erc  "
720  PRINT"     COUNTS ";"     TOTALS ";"     COUNTS   ";" (/min)  "
730 PRINT"     (cpm)  ";"     (cpm) ";"     (cpm)    "
740  FOR N=1 TO E
750    PRINT TAB(5);A(N);TAB(17);B(N);TAB(28);D(N);TAB(40);P(N)
760  NEXT
770  PRINT
780  PRINT
790  PRINT"TISSUE COUNTS  ";C;" cpm";"     TISSUE WEIGHT  ";W;" mg"
800  PRINT
810  VDU3
820  @%=10
830  PRINT
840  PRINT:PRINT:PRINT:PRINT
850  INPUT"DO YOU WANT THE EFFLUX CURVE PLOTTED (ON THE SCREEN)?(Y/N)"YES3$
860  IF YES3$="Y" THEN 940
870  PRINT:@%=10:VDU3
880  PRINT"DO YOU HAVE FURTHER DATA?(Y/N)"
890  INPUT A$
900  IF A$="Y" THEN 10
910  PRINT"THE END"
920  END
930
940  REM     CURVE PLOTTING
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```
 950  MODE4
 960  VDU24,0;0;1279;1023;
 965  VDU28,11,1,39,0
 970  X=20
 980  XLENGTH=1100+100
 990  MOVE 100,100:DRAW XLENGTH,100
1000  MOVE 100,100:DRAW 100,940
1010  FOR Y=1 TO 4
1020    MOVE 100,Y*210+100:DRAW 116,Y*210+100
1030  NEXT Y
1040  FOR N=1 TO E
1050    MOVE 100+(T*X)+(R(N)*X),100
1060    DRAW 100+(T*X)+(R(N)*X),80
1070    MOVE (T+R(1))*X+100,(P(1)*7000)+100
1075  NEXT N
1080  FOR N=2 TO E
1090    DRAW (T+R(N))*X+100,(P(N)*7000)+100
1100  NEXT N
1110  VDU5
1120  PLOT 4,0,1000:PRINT"erc(/min)"
1130  PLOT 4,0,952:PRINT".12"
1140  PLOT 4,0,742:PRINT".09"
1150  PLOT 4,0,532:PRINT".06"
1160  PLOT 4,0,322:PRINT".03"
1170  PLOT 4,0,112:PRINT".00"
1180  Z=R(E) DIV 10
1190  FOR N=1 TO Z
1200    MOVE 100+(N*X*10),100
1210    DRAW 100+(N*X*10),70
1215    PRINT;
1220    PRINT;N*10
1230  NEXT N
1240  PLOT 4,425,32:PRINT"TIME(min)"
1250  VDU4
1260  PRINT"PRINT GRAPH ";:INPUT GRAPH$
1270  PRINT STRING$(14," ")
1280  PRINT"erc   :  PER TIME PERIOD"
1290
1300  IF GRAPH$="Y" OR GRAPH$="y" PROCSDUMP ELSE GOTO 1330
1310  PRINT"PRESS SPACE BAR TO CONTINUE"
1320  REPEAT UNTIL GET =32
1330  PRINT
1340  @%=10
1350  VDU3
1360  MODE7:GOTO 880
1370  DEF PROCSDUMP
1380 *SDUMP
1390  ENDPROC
```